# SOLUBLE RECEPTORS FOR ADVANCED GLYCATION END PRODUCTS AS PREDICTORS OF RESTENOSIS FOLLOWING PERCUTANEOUS CORONARY INTERVENTION

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**By**

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## ABSTRACT

The principal cause of non-ST-segment myocardial infarction (NSTEMI), a subclass of acute coronary syndrome (ACS), is thrombosis and the underlying cause is atherosclerosis. Percutaneous coronary intervention (PCI) is one of the treatments to attenuate the ischemic effects of severe coronary artery stenosis. However, restenosis following PCI (post-PCI) is a major problem for the long-term success of the procedure. Recently, the interaction of advanced glycation end products (AGE) with the receptor for advanced glycation end products (RAGE) has been implicated in the development of atherosclerosis in animal models. Interaction of AGE with RAGE results in activation of nuclear factor kappa-B (NF-κB), release of cytokines including tumor necrosis factor-alpha (TNF-α), the expression of adhesion molecules including soluble vascular adhesion molecule-1 (sVCAM-1) and induction of oxidative stress all of which have been implicated in the development of atherosclerosis. The soluble receptor for advanced glycation end products (sRAGE) acts as a decoy for RAGE ligands (AGEs) and this occurs by competing with RAGE. In animal models, balloon inflation and de-endothelialization of the carotid artery increase the concentration of AGE and RAGE in the arterial wall and induces neointimal hyperplasia and stenosis. Treatment with sRAGE in animal models reduces neointimal growth and decreases smooth muscle cell migration and proliferation and expression of extracellular matrix.

It is hypothesized that NSTEMI and post-PCI restenosis may be due to low levels of serum sRAGE resulting in increased AGE and RAGE interactions. Low levels of sRAGE would also increase the levels of serum TNF-α and sVCAM-1.

The objectives of this study were to determine whether: (1) the levels of serum sRAGE are lower and the levels of AGE, TNF-α and sVCAM-1 are higher in NSTEMI patients compared to control; (2) the levels of serum sRAGE are lower and the levels of AGE, TNF-α and sVCAM-1 are higher in NSTEMI patients with restenosis compared to those without restenosis; and (3) sRAGE or AGE/sRAGE ratio may serve as a biomarker/ predictor of NSTEMI and post-PCI restenosis.

The study objectives include 46 consecutive NSTEMI patients undergoing elective PCI and 28 healthy age-matched male controls. Pre-PCI and 6 month post-PCI angiography were performed in all NSTEMI patients. Blood samples were collected at designated intervals for the measurement of sRAGE, AGE, TNF-α, and sVCAM-1 using commercially available enzyme-linked immunosorbent assay (ELISA) kits.

The levels of serum sRAGE were lower and those of TNF-α, sVCAM-1, AGE and AGE/sRAGE were higher in NSTEMI patients compared to control subjects. (sRAGE, 884.55 ± 50 vs. 1287 ± 41.5 pg/mL{$p<0.001$}; TNF-α, 23.1 ± 2.3 vs. 10.3 ± 0.8 pg/mL {$p<0.002$}; sVCAM-1, 1059.62 ± 70.8 vs. 651 ± 35.5 ng/mL {$p<0.0003$}, AGE, 1192.50 ± 82.6 vs. 669.40 ± 47.9 ng/mL {$p<0.001$}; and AGE/sRAGE, 1.75 ± 0.17 vs. 0.52 ± 0.06 {$p<0.001$}).

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV) and accuracy of the sRAGE biomarker test were 59%, 100%, 100%, 100% and 74% respectively, while those of the AGE/sRAGE test were 85%, 91%, 97%, 67%

and 86%, respectively, in the diagnosis of patients with NSTEMI acute coronary syndrome.

The pre-PCI levels of serum sRAGE in patients with restenosis were significantly lower ($p<0.001$) than in patients without restenosis (610.6 ± 24.1 vs. 1143.8 ± 52.5 pg/mL). The post-PCI levels of serum sRAGE were significantly lower ($p<0.0001$) in patients with restenosis compared to those without restenosis (477 ± 18.6 vs. 1106.7 ± 41.9 pg/mL). The pre-PCI levels of serum TNF-α and sVCAM-1 were significantly higher ($p<0.001$ and $p<0.001$) in patients with restenosis compared to those without restenosis (TNF-α, 37.9 ± 2.5 vs. 11.6 ± 0.41 pg/mL; sVCAM-1, 1381.8 ± 63.5 vs. 811.37 ± 26.5 ng/mL). The post PCI levels of serum TNF-α and sVCAM-1 were also significantly higher ($p<0.0001$ and $p<0.0001$) in patients with restenosis than in patients without restenosis (TNF-α, 48.4 ± 1.4 vs. 12.5 ± 0.44 pg/mL; sVCAM-1, 1381.8 ± 63.5 vs. 762.2 ± 26.4 ng/mL). The post-PCI levels sRAGE were lower while those of TNF-α and sVCAM-1 were higher compared to the pre-PCI levels in patients with restenosis. However, the pre- and post-PCI levels of serum sRAGE, TNF-α and sVCAM-1 were similar in patients without restenosis. The pre-PCI levels of serum AGE and AGE/sRAGE were significantly higher ($p<0.001$ and $p<0.001$) in patients with restenosis compared to those without restenosis (AGE, 1512.1 ± 84.53 vs. 891.7 ± 92.4 ng/mL; 2.39 ± 0.20 vs. 1.03 ± 0.17).

The sensitivity, specificity, PPV, NPV, and accuracy of the pre-PCI sRAGE tests were 73%, 100%, 100%, 80%, and 87%, respectively while those of the AGE/sRAGE tests were 81%, 94%, 93%, 84% and 88%, respectively in identifying patients with post-PCI restenosis.

In conclusion the results suggest that: (1) the levels of serum sRAGE are lower while those of TNF-α, sVCAM-1, AGE and AGE/sRAGE are higher in NSTEMI patients compared to control subjects; (2) serum levels of sRAGE are negatively correlated with the number of diseased vessels; (3) Both low sRAGE and high AGE/sRAGE may serve as a biomarker/predictor of NSTEMI, but AGE/sRAGE has a greater sensitivity compared to sRAGE; (4) the pre-PCI levels of serum sRAGE are lower while those of AGE/sRAGE are higher in patients with restenosis compared to those without restenosis; and (5) both low sRAGE and high AGE/sRAGE may serve as a predictor/ biomarker of post-PCI restenosis; however, AGE/sRAGE has a greater sensitivity than sRAGE.

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## DEDICATION

I dedicate this thesis to my parents Reuben and Dorothy McNair. You always belived in my abilities and directed me to strive towards success by using the tools of education, dicipline and hard work. You set the example by achieving success within your own lives and then gave me the opportunity to excel by instilling in me the belief that "anything is possible if you put your mind to it". Words cannot express my gratitude for having you as parents.

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## LIST OF ABBREVIATIONS

| Symbol | Full Name |
|---|---|
| α4β1 | very late antigen-4 |
| α4β2 | alpha four beta 2 |
| ACAT | acyl: cholesterol acyltransferace |
| ACEi | angiotensin converting enzyme inhibitor |
| Acetyl COA | acetyle co-enzyme A |
| ACh | acetylcholine |
| ACC | American College of Cardiology |
| ACS | acute coronary syndrome |
| ADP | adenosine diphosphate |
| AGE | advanced glycation end product |
| AHA | American Heart Association |
| ALS | amyotrophic lateral sclerosis |
| αMβ2 | macrophage-1 antigen |
| AMP | adenosine monophosphate |
| ARB | angiotensin receptor blocker |
| ASA | aspirin |
| ATP | adenosine triphosphate |
| αX | complement component 3 receptor 4 subunit |
| BCL-3 | B-cell CLL/lymphoma 3 |
| bFGF | basic fibroblast growth factor |
| BHT | butylated hydroxyanisole |
| BMI | body mass index |

| | |
|---|---|
| BMS | bare metal stent |
| $Br^-$ | bromide anion |
| BP | blood pressure |
| BPD | balloon protection device |
| C | cysteine domain in a chemokine |
| C4 | complement receptor |
| $C_3$ | complement 3 |
| $C_5$ | complement 5 |
| $Ca^{2+}$ | calcium ion |
| CABG | coronary artery bypass graft |
| CAD | coronary artery disease |
| CBF | coronary blood flow |
| C-C | subfamily of chemokine in which the first two cysteines are adjacent to one another |
| CCL2 | same as monocyte chemoattractant protein-1 |
| CCL3 | same as monocyte inflammatory protein-1 alpha |
| CCL4 | same as monocyte inflammatory protein-1 beta |
| $CCl_4$ | carbon tetrachloride |
| CCL5 | same as RANTES |
| CCL8 | chemokine ligand 8 |
| CD8+ T cells | cytotoxic T cells or killer T cells |
| CD11a | one of two components to form LFA-1 (CD 11/ CD18) |
| CD11b | cluster of differentiation molecule 11b |
| CD11c | integrin alpha X |

| | |
|---|---|
| CD 11/ CD18 | lymphocyte function-associated antigen1 |
| CD29 | integrin ligand 29 |
| CD31 | same as platelet endothelial cell adhesion molecule-1 |
| CD62 | integrin ligand 62 |
| CD64 | integrin ligand 64 |
| CD66 | integrin ligand 66 |
| CD67 | integrin ligand 67 |
| CFR | coronary flow reserve |
| CHD | coronary heart disease |
| CHF | congestive heart failure |
| $Cl^-$ | chloride anion |
| CML | 3, 4-N-ε-(carbomethyl) lysine |
| CMV | cytomegalovirus |
| c-Myc | gene which regulates other genes |
| Cox-2 | cyclo-oxygenase-2 |
| C. pneumonia | Chlamydophila pneumonia |
| CVD | cardiovascular disease |
| c-Rel | reticuloendotheliosis viral oncogene homolog-c |
| CR3 | complement receptor 3 |
| CRP | C-reactive protein |
| CSF | colony stimulating factor |
| $Cu_{2+}$ | cuprous |
| C-X-C | subfamily of chemokine in which an amino acid residue separates the first two cyteines. |

| | |
|---|---|
| CXCCL-16 | CXC chemokine |
| $CX_3C$ | subfamily of chemokines |
| $CX_3CL$-1 | fractalkine (chemokine) |
| $CX_3CR$ | receptor for $CX_3C$ chemokines |
| CXCL-8 | same as IL-8 |
| DCA | directional coronary atherectomy |
| DD | double distilled |
| DFO | desferrioxamine |
| DES | drug eluting stent |
| DM | diabetes mellitus |
| DNA | deoxyribonucleic acid |
| %DS | percent diameter stenosis |
| DTT | dithiothreitol |
| ECF | extracellular fluid |
| ECG | electrocardiogram |
| EDTA | ethylenediamine tetraacetic acid |
| EDRF | endothelial-derived relaxing factor |
| EGF | epidermal growth factor |
| EIA | enzyme immunoassay |
| ELAMs | endothelial leukocyte adhesion molecules |
| ELISA | enzyme-linked immunosorbentassay |
| ELKS | glutamic acid (E), leucine (L) lysine (K), serine (S) |
| ELR + | CXC chemokine with aglutamine-leucine arginine motif |
| ELR- | chemokines that lack the ELR motif |

| | |
|---|---|
| eNOS | endothelial nitric oxide synthetase |
| E1 ligase | ubiquitin activating enzyme |
| E2 ligase | ubiquitin conjugating enzyme |
| E3 ligase | ubiquitin enzyme |
| esRAGE | extracellular soluble RAGE |
| ETT | exercise tolerance test |
| $Fe^{2+}$ | ferrous fumarate |
| $Fe^{3+}$ | ferric fumarate |
| FFR | fractional flow reserve |
| FGF | fibroblast growth factor |
| FPIA | fluorescence polarization immunoassay |
| G | guanine |
| $G_1$ | growth cycle 1: phase for preparation of chromosomes |
| $G_1$ | type of GTP binding protein |
| $G_2$/M | growth cycle$_2$/M: phase for preparation of mitosis |
| GlyCAM-1 | glycosylation-dependent cell adhesion molecule |
| GM-CSF | granulocyte-monocyte-colony stimulating factor |
| GP | glycoprotein |
| gp91ds-tat | chimeric peptide inhibitor |
| GSH | reduced glutathione |
| GSH-$P_x$ | glutathione peroxidase |
| GTP | guanine triphosphate |
| $H^+$ | hydrogen ion |
| HAEC | human aortic endothelial cells |

| | |
|---|---|
| $HbA_1$ | glycated hemoglobin |
| HB-EGF | heparin-binding growth factor |
| HCl | hydrochloric acid |
| HDL-C | high-density lipoprotein-cholesterol |
| $H_2O$ | water |
| $H_2O_2$ | hydrogen peroxide |
| HMEC | human microvascular endothelial cells |
| HMG-CoA | 3-hydroxy-3-methylglutaryl Co enzyme A |
| HOCl | hypochlorous acid |
| $HOO^{\cdot}$ | hydroperoxyl radical |
| HPAEC | human pulmonary aortic endothelial cells |
| HPMC | human peritoneal mesothelial cells |
| HSP | heat shock proteins |
| HTN | hypertension |
| HUVEC | human umbilical vein endothelial cell |
| $I^-$ | iodide anion |
| ICAM-1 | intracellular adhesion molecule-1 |
| ICAM-2 | intracellular adhesion molecule-2 |
| ICAM-3 | intracellular adhesion molecule-3 |
| IFN | interferon |
| IFN-γ | interferon-gamma |
| IgA | immunoglobulin A |
| IGF-I | insulin growth like factor I |
| IgG | immunoglobulin G |

| | |
|---|---|
| IgSF | immunoglobulin super family |
| IHD | ischemic heart disease |
| IκB | inhibitor of kappa B |
| IκBα | inhibitor of kappa B alpha |
| IKK | IκB kinase |
| IKKAP1 | IKK-associated protein 1 |
| IKKγ | IKK gamma |
| ISR | in-stent restenosis |
| κD | kilo dalton |
| IL | interleukin |
| IL-1 | interleukin-1 |
| IL-1β | interleukin-1β |
| IL-2 | interleukin-2 |
| IL-5 | interleukin-5 |
| IL-6 | interleukin-6 |
| IL-7 | interleukin-7 |
| IL-8 | interleukin-8 |
| IL-10 | interleukin-10 |
| IL-12 | interleukin-12 |
| IL-1Ra | blocks the receptor for IL-1 |
| IL-1RACP | IL-1R accessory protein |
| IL-1R1 | interleukin-1 receptor, type 1 |
| INT-violet | intensely purple formazan dye |
| IRAK | IL-1R-associated kinase |

| | |
|---|---|
| ISR | in-stent restenosis |
| IVUS | intravascular ultrasound |
| $L^{\cdot}$ | lipid radical |
| LAD | leukocyte adhesion deficiency syndrome |
| LAST | late angiographic stent thrombosis |
| LDL | low-density lipoprotein |
| LDL-C | low-density lipoprotein-cholesterol |
| LFA-1 | lymphocyte function-associated antigen 1 |
| LOOH | lipid hydroperoxides |
| Lp (a) | lipoprotein (a) |
| LPL | lipoprotein lipase |
| LPS | lipopolysaccharides |
| $LTB_4$ | leukotriene $B_4$ |
| MAC-1 | macrophage-1 antigen (consitsing of CD11b and CD18) |
| MACE | major cardiac events |
| MAPKKK | mitogen activating protein kinase kinase kinase |
| MCP-1 | monocyte chemotactic protein-1 |
| M-CSF | macrophage-colony stimulating growth factor |
| MDA | malondialdehyde |
| MI | myocardial infarction |
| MIP1-α | monocyte inflammatory protein-1 alpha |
| MIP1-β | monocyte inflammatory protein- 1 beta |
| MLD | minimum lumen diameter |

| | |
|---|---|
| mm | millimeter |
| MMLDL | minimally modified low-density lipoprotein |
| MMP | metalloproteinase |
| mPGES-1 | microsomal prostaglandin synthetase-1 |
| MPO | myeloperoxidase |
| mRNA | messenger ribonucleic acid |
| MyD88 | myeloid differentiation primary response gene (88) |
| NAC | N-acetyl cysteine |
| NADH | nicotinamide adenine dinucleotide hydrogenase |
| NADP | nicotinamide adenine dinucleotide phosphate |
| NADPH | reduced nicotinamide adenine dinucleotide phosphate |
| NAP-1 | neutrophil activating protein-1 |
| NEMO | NF-κB essential modulator |
| NFIL-6 | nuclear factor IL-6 |
| NF-κB | nuclear factor kappa B |
| NF-κB / Rel | nuclear factor kappa B/ reticuloendotheliosis viral oncogene homolog |
| $NH_2$ | amido group |
| NIK | NF-κB interacting protein |
| NK | natural killer cells |
| $NO^{\cdot}$ | nitric oxide |
| $NO_2$ | nitrogen dioxide |
| $N_2O_3$ | nitrogen trioxide |
| NPV | negative predictive value |

| | |
|---|---|
| NSTE | non-ST segment elevation |
| NSTEMI | non-ST segment elevationmyocardial infarction |
| $^1O_2$ | singlet oxygen |
| $O_2$ | molecular oxygen |
| $O_2^{\cdot -}$ | superoxide anion |
| OCl | hypochlorite |
| OFR | oxygen free radical |
| $OH^{\cdot}$ | hydroxyl radical |
| OH | hydroxide |
| OR | oxygen radical |
| $ONOO^-$ | peroxynitrite |
| OxLDL | oxidized low-density lipoprotein |
| p100 | protein cellular coactivator |
| p150 | CD11c |
| p105 | nuclear factor kappa light chain gene enhancer in B cells |
| PAF | platelet activating factor |
| PAI-1 | plasminogen activator inhibitor-1 |
| PC | phosphocholine |
| PCI | percutaneous coronary intervention |
| PCR | polymerase chain reaction |
| PECAM-1 | platelet endothelial cell adhesion molecule |
| PD | peritoneal dialysis |
| PDGF | platelet-derived growth factor |
| $PGG_2$ | prostaglandin $G_2$ |

| | |
|---|---|
| $PGH_2$ | prostaglandin $H_2$ |
| PPV | positive predictive value |
| PSGL-1 | P-selectin glycoprotein ligand-1 |
| PMNL | polymorphonuclear leukocyte |
| PTA | percutaneous transluminal angioplasty |
| PTCA | percutaneous transluminal coronary angioplasty |
| PTDC | pyrrolidine dithiocarbamate |
| PUFA | polyunsaturated fatty acid |
| QCA | quantitative coronary coronary angiography |
| RA | rotational atherectomy |
| RANTES | regulated on activation normal T cell expressed and secreted |
| RAGE | receptor for advanced glycation end products |
| RASMC | rat aortic smooth muscle cell |
| Rel A | reticuloendotheliosis viral oncogene homolog A |
| Rel B | reticuloendotheliosis viral oncogene homolog B |
| RIP | receptor interacting protein |
| RNA | ribonucleic acid |
| $RO^{\cdot}$ | alkoxyl radical |
| $ROO^{\cdot}$ | peroxyl radical |
| ROS | reactive oxygen species |
| RPM | revolutions per minute |
| RVD | reference vessel diameter |
| sICAM | soluble intercellular adhesion molecule |

| | |
|---|---|
| SDS | sodium lauryl sulphate |
| SEM | standard error of the mean |
| $sLe^x$ | sialyl Lewis x (carbohydrate ligand) |
| $sLe^a$ | sialyl Lewis a (carbohydrate ligand) |
| SMC | smooth muscle cell |
| SOD | superoxide dismutase |
| S phase | growth cycle phase for synthesis of DNA |
| SPSS | statistical program for the social sciences |
| SEM | standard error of the mean |
| sRAGE | soluble receptor for advanced glycation end products |
| STE | ST segment elevation |
| STEMI | ST segment elevation myocardial infarction |
| sTNF | soluble tumor necrosis factor-alpha |
| sVCAM | soluble vascular cell adhesion molecule |
| SVG | saphenous vein graft |
| TACE | TNF-α converting enzyme |
| TBA | thiobarbituric acid |
| TC | total cholesterol |
| tHcy | total homocysteine |
| TIMI | thrombolytics in myocardial infarction |
| TNF-α | tumor necrosis factor-alpha |
| TNF-β | tumor necrosis factor-beta |
| TNFR1 | tumor necrosis factor receptor 1 |
| TNFR2 | tumor necrosis factor receptor 2 |

| | |
|---|---|
| TG | triglycerides |
| TGF-α | transforming growth factor-alpha |
| TGF-β | transforming growth factor-beta |
| TLR | target lesion revascularization |
| tPA | transplasminogen activator |
| TRADD | TNFR1-associated death domain protein |
| TRAF2 | TNFR-associated factor 2 |
| TRAF6 | TNFR-associated factor 2 |
| TTFA | thenoyltriflouroacetone |
| 26 S Proteasome | twenty-six Svedberg sedimentation coefficient proteasome |
| $TxA_2$ | thromboxane $A_2$ |
| U/A | unstable angina |
| VEGF | vascular endothelial growth factor |
| VCAM-1 | vascular adhesion molecule-1 |
| VLA-4 | very late antigen-4 |
| VLDL | very low-density lipoprotein |
| VLDL-TG | very low-density lipoprotein-triglyceride |
| VSMC | vascular smooth muscle cell |
| V/V | volume to volume |
| X | amino acid residues in a chemokine |
| 1VD | one vessel disease |
| 2VD | two vessels disease |
| 3VD | three vessels disease |

## 1.0 INTRODUCTION

Advanced glycation end products (AGE) are a heterogeneous group of irreversible adducts resulting from non-enzymatic glycation and oxidation of proteins, lipids and nucleic acids (Thorpe and Baynes, 2003; Prasad, 2006). AGE act on receptors for advanced glycation end products (RAGE). There are three forms of RAGE (Thorpe and Baynes, 2003; Prasad, 2006; Yonekura et al., 2003; Hudson et al., 2005): Full-length (transmembrane receptor), N-truncated (membrane bound) and C-truncated (soluble receptors for AGEs {sRAGE. The interaction of full-length RAGE with AGEs leads to increased expression of adhesion molecules, including soluble vascular cell adhesion molecule-1 (sVCAM-1) and the release of the cytokine tumor necrosis factor-alpha (TNF-α) (Prasad, 2006; Hofmann et al., 1999; Reznikov et al., 2004). RAGE-AGE interaction also leads activation of nuclear factor kappa B (NF-κB) (Hofmann et al., 1999) which in turn leads to increased expression of proinflammatory genes for adhesion molecules and cytokines (Prasad, 2006), and generation of oxygen radicals (Yan et al., 1994; Wautier et al., 2001) and up-regulation of itself. The function of the N-truncated RAGE is poorly understood. sRAGE circulates in the plasma (Yonekura et al., 2003) and acts as a competitive receptor (receptor that binds a ligand preventing it from binding to its normal receptor) for RAGE ligands, competing with full-length RAGE for ligand binding (Geroldi et al., 2006a). It has a protective role by preventing the activation of full-length RAGE that could otherwise result in enhanced oxidative stress and activation of NF-κB, cytokines and adhesion molecules leading to tissue damage and the development of atherosclerosis. Adhesion molecules, cytokines and oxygen radicals are involved in atherosclerosis, progression of lesion and lesion instability (Libby et al., 2006; Young et al., 2006; Prasad, 1999a; Blankenberg et al.,

2003). The AGE and RAGE axis has been implicated in the pathogenesis of atherosclerosis in diabetes (Schmidt et al., 2000; Schmidt, 1999).

Percutaneous coronary intervention (PCI) has become the treatment of choice to reduce the ischemic effects of severe coronary artery disease, with over 1 million interventions achieved around the world each year (Aronson, 2002). Although much progress has been made in the field of interventional cardiology, restenosis is a major problem for long-term success after PCI such as angioplasty and stenting (Kastrati, et al., 2001). Restenosis following PCI is associated with neointimal hyperplasia. Balloon injury in carotid and arterial de-endothelialization in animal models increase the levels of RAGE and AGE in the arterial wall and produce neointimal hyperplasia (Zhou et al., 2003; Sakaguchi et al., 2003). Treatment with sRAGE in animal models reduced neointimal growth, decreased smooth muscle proliferation and migration, and expression of extracellular matrix (Zhou et al., 2003; Sakaguchi et al., 2003). In addition, sRAGE attenuates the atherosclerotic lesion in diabetic apo-E $^{-/-}$ mice and this is associated with decreases in aortic sVCAM-1, tissue factor and matrix metalloproteinase-9 (MMP-9) (Wendt et al., 2000).

The principal cause of acute coronary syndrome (ACS) is thrombosis and the underlying cause is atherosclerosis. Acute coronary syndrome includes ST-segment elevation myocardial infarction (STEMI), non ST-segment elevation myocardial infarction (NSTEMI) and unstable angina (U/A) (American Heart Association, 2008). ACS may be due to an imbalance between RAGE, AGE, and sRAGE. Post-PCI restenosis may be due to low levels of serum sRAGE in patients (Falcone et al., 2005; Pulleritis et al., 2005; Geroldi et al., 2005) However, it is not known whether: (1) levels of serum

sRAGE are low in patients with NSTEMI and (2) pre-PCI levels of serum sRAGE are lower in patients who develop post-PCI restenosis compared to those who do not.

Since the combination of AGE, RAGE, and sRAGE determines the extent of vascular injury, the measurements of these parameters would be relevant in determining the vascular injury (atherosclerosis) (Zhou et al., 2003; Prasad 2006a). However, in humans' RAGE is inaccessible to measurement since it is a cell surface protein on the vascular endothelium. Thus, the levels of serum sRAGE and the ratio of AGE/sRAGE may be used as a biomarker/predictor of vascular complications.

This study proposes to investigate whether: (1) low levels of serum sRAGE and a high ratio of AGE/sRAGE are biomarkers/ predictors of NSTEMI (ACS) and post-PCI restenosis and (2) low levels of serum sRAGE or high levels of AGE/sRAGE is a better biomarker/ predictor of NSTEMI and post-PCI restenosis.

## 1.1 REVIEW OF THE LITERATURE

### 1.2 Atherosclerosis and Coronary Artery Disease

The term atherosclerosis is derived from the Greek “athero” (gruel or porridge) and “sclerosis” (hardening). It is a chronic inflammatory disease of the arteries in which macrophages and oxidized lipids are the primary agents that cause intimal fibro-fatty plaque formation and progression. Atherosclerosis begins in childhood and slowly progresses over many years before it manifests itself with clinical symptoms (Ross and Glomset, 1976). The arteries generally affected by atherosclerosis are the thoracic aorta, coronary, internal carotids, femoral, basilar, vertebral, and circle of Willis. Myocardial infarction and stroke are two of the major clinical consequences of diminished or halted blood flow created by atherosclerosis (Ross and Glomset, 1976; McNair et al., 2006).

#### 1.2.1 Epidemiology of Atherosclerosis

In North America, atherosclerosis continues to be one of the prominent causes of morbidity and mortality. Prasad (2003) states “The incidence of disease related to atherosclerosis is as follows: ischemic heart disease (IHD), 7 million; peripheral vascular disease, 3 million; and stroke, 0.75 million per year”. It is a ubiquitous disease in developed countries and has geographical variations. For instance, coronary artery disease in North America is six times higher than in Japan (Ross, 1986; McNair, 2006).

#### 1.2.2 Pathology of Atherosclerosis

Intimal focal lesions of atherosclerosis are visible fatty streaks with raised yellow areas that are slender and longitudinally oriented. They are made up of lipid-laden foam cells that are macrophages and/or smooth muscle cells. Fibrous plaque lesions are raised off-

white lesions with three layers. The first layer is a fibrous cap consisting of SMC and leukocytes, along with dense connective tissue. This connective tissue consists of a basement membrane, collagen fibrils, proteoglycans, and elastin. The second layer is a cellular region under and adjacent to the fibrous cap. This layer is composed of smooth muscle cells, T-lymphocytes and macrophages. The final lower necrotic layer is composed of large foam cells, cellular debris, lipids, and calcium crystals (Duff and McMillian, 1951). However, most patients with atherosclerosis present with a combination and/or variations of each of these characteristics. The organization of lipid and connective tissue in these lesions determines whether or not they are "complicated" lesions that are stable or unstable with the possibility of rupture and, or, thrombosis (Prasad, 2000a). Nevertheless, the clinical event that arises from coronary atherosclerotic plaque formation is myocardial ischemia (decreased coronary perfusion). According to Preuss et al. (1987), there are three major types of ischemia: (1) tolerable ischemia, which has a low degree of reduced blood flow; (2) critical ischemia, which reflects a modestly severe flow reduction; and (3) lethal ischemia, which has a severe flow reduction and is the limit of myocardial survival. Lethal ischemia, if left untreated, can lead to irreversible cell necrosis or myocardial infarction (MI) (McNair et al., 2006).

### 1.2.3 Acute Coronary Syndrome (ACS)

Acute coronary syndrome is a classification of clinical symptoms associated with acute myocardial ischemia (CAD) at rest and is the result of complete or partial occlusion of a coronary artery by a thrombus consisting of platelets and fibrin (American Heart Association, 2008). ACS patients may be categorized on the basis of the 12-lead electrocardiogram (ECG) with either ST segment elevation (STE) or non-ST segment elevation (NSTE). The diagnosis of ACS with myocardial infarction (MI) is based upon

evidence of a raised plasma troponin level. Troponins are proteins that are associated with myocardial muscle damage. Patients with ACS that have STE with an acute rise in troponins are termed STEMI, whereas patients with non-ST-elevation and an acute rise in troponins are considered as NSTEMI. However, those patients with NSTE without an elevation in troponins are considered to have unstable angina (U/A). Patients with STEMI have a greater risk of early mortality compared to those with NSTEMI. Moreover, those patients with STE ACS who have a total thrombotic occlusion of a coronary artery usually require immediate therapy to improve myocardial perfusion. The therapy is typically accomplished by opening the occlusion, utilizing fibrinolytic administration and/or PCI. The goal of PCI is to increase coronary blood flow and reduce the risk of infarction in the case of NSTE and U/A and decrease the risk of re-infarction in STEMI and NSTEMI (American Heart Association, 2008; Spinler, 2007). A study conducted by the American College of Cardiology/American Heart Association (ACC/AHA), involving 6923 patients from 2002 to 2006, known as the CRUSADE initiative (Can Rapid Risk Stratification of Unstable Angina Patients Suppress Adverse Outcomes with Early Implementation of the ACC/AHA Guidelines), was to assess and advance adherence to 2002 ACC/AHA guidelines for the care of patients with unstable angina and NSTEMI who underwent PCI. One of the early findings of the CRUSADE registry identified the acute administration of pharmacological therapy as an area that needed improvement. CRUSADE data showed that the frequencies of aspirin use, beta-blockers, heparin, glycoprotein (GP) IIb/IIIa inhibitors, and clopidogrel for acute therapy without contraindications were 96%, 92%, 87%, 45%, and 60%, respectively. However, the ACC/AHA guidelines recommended the administration of GP IIb/IIIa inhibitors together with aspirin and heparin for all patients prior to undergoing coronary

angiography and PCI. Additionally, with the exception of those with an increased risk for bleeding, clopidogrel is suggested by the ACC/AHA for all NSTE ACS patients, particularly those undergoing PCI (Braunwald et al., 2002). Concerning discharge medications, the CRUSADE data through 2006 revealed that aspirin and beta-blockers were utilized at the time of discharge by 96% and 94% of patients respectively, not having contraindications. In addition, lipid-lowering drugs were used by 90% of patients with hyperlipidemia (increased total cholesterol or low-density lipoprotein levels) without contraindications (Schwartz et al., 2001). The percentage of suitable patients receiving clopidogrel as acute therapy was lower than the percentage of patients receiving this drug at the time of discharge (73%), perhaps demonstrating a large proportion of patients undergoing PCI. Angiotensin-converting enzyme inhibitors (ACEi) are indicated for those patients with an ejection fraction less than 40%, congestive heart failure (CHF), diabetes mellitus, or hypertension. Related to discharge, ACE inhibitors were administered in 65% of the patients with no contraindications, contrary to the recommendations from the ACC/AHA to use these medications in most patients with CAD (Smith et al., 2006). Consistency with the 2002 ACC/AHA guidelines between 2002 and 2004 showed an increase in adherence in acute therapies (from 30 to 40%) and discharge therapies (from 30 to 50%) (Spinler, 2007). Perhaps these adjustments display the time necessary to integrate new guidelines into practice. The evolving evidence-based guidelines for the treatment of ACS will ultimately enhance patient outcomes. For the purposes of this study only patients' with ACS NSTEMI were enrolled.

### 1.2.4 Cytokines

In order to fully understand the complexities associated with the genesis and progression of atherosclerosis, one must have a general comprehension of the chemical mediators associated with this disease. Cytokines play a major role in the initiation and development of atherosclerosis and coronary artery disease.

In general, cytokines are soluble low molecular weight (7 to 40 κD) structurally related proteins that regulate the function of other cell forms (Mitchell and Cotran, 2003). Historically, they were understood to be an integral part in cellular immune responses (Weissman, 1992). However, it has since been demonstrated that cytokines function in the modulation of acute and chronic inflammation by binding to specialized endogenously and exogenously controlled receptors on target cells (McEver and Cummings, 1997). These transient multifunctional proteins are pleotrophic and may induce the production or influence the activity of additional cytokines. The cytokines are mainly produced by activated macrophages and lymphocytes, and secondarily by other cell types such as endothelial and connective tissue cells (Mitchell and Cotran, 2003).

#### 1.2.4.1 Effects of Cytokines

Cytokines regulate cell function by six mechanisms: (1) *Paracrine Effects* are expressed when chemical mediators/ growth modulators act upon other cell types in the area, such as occurs when marrow stromal cells form IL-7 which then induces the differentiation of marrow B-cell progenitors; (2) *Autocrine Effects* are manifest when chemical mediators/ growth modulators act upon the cells that have produced them, e.g. when IL-2 stimulates T cells by binding to high affinity IL-2 receptors; and (3) *Endocrine Effects* take place

when cytokines control distant cells by circulating systemically, e.g. when IL-1 and TNF induce the acute phase response in the course of inflammation (Denholm and Lewis, 1987; Mitchell and Kumar, 2003). Another example of endocrine effects of cytokines occurs when IL-6 released at a local inflammatory region causes increased liver production of acute phase protein (McInnes, 2003); (4) *Amplification Effects* of cytokines are observed when IL-1 production by TNF stimulation causes IL-6 synthesis (Mitchell and Kumar, 2003); (5) *Antagonistic Effects* induced by cytokines can control the degree and kind of response. Examples of the antagonistic effects of cytokines are the inhibition of macrophages by IL-10 and the stimulation of macrophages by IFN-γ (Mitchell and Kumar, 2003); (6) *Pleiotropic Effects* are induced by cytokines, in which many different effects are elicited from one particular cytokine.

1.2.4.2 Types of Cytokines

Cytokines have been categorized according to their principal functions or by the characteristics of their target cells.

(1) Cytokines that dictate lymphocyte control: This class of cytokines controls lymphocytes by stimulation, growth and differentiation. Cytokines may have positive effects when they interact with IL-2 and IL-4, promoting lymphocyte growth. Negative effects occur with the combination of IL-10 and transforming growth factor-beta (TGF-β), which inhibits the immune response (Mitchell and Kumar, 2003).

(2) Cytokines that participate in natural immunity: The four major inflammatory cytokines in this category are TNF-α, IL-1β, IL-6 and the interferons–alpha and beta (IFN-α and β) (Mitchell and Kumar, 2003).

(3) Cytokines that stimulate inflammatory cells: Macrophages are stimulated through cell-mediated immune responses by the cytokines interferon-gamma (IFN-γ), TNF-α, lymphotoxin (tumor necrosis factor- β), IL- 5, IL-10, and IL-12 (Mitchell and Kumar, 2003).

(4) Chemokines: Low molecular weight regulatory proteins with analogous amino acid sequences of the cytokine super family which stimulate recruitment of particular leukocyte subsets (Adams and Lloyd, 1997; Graves and Jiang, 1995).

(5) Cytokines that induce hematopoiesis: These cytokines are named colony stimulating factors, due to their historical classification which is based upon their stimulation of growth of hematopoietic cell colonies from bone marrow precursors (Mitchell and Kumar, 2003).

1.2.4.2.1 Chemokines: A Major Cytokine in Atherosclerosis

Historically, the development of a plausible theory for the recruitment of a specific type of leukocyte into an inflamed tissue remained an enigma. Recently, a better knowledge of various forms of inflammation has evolved subsequent to the recognition of chemotactic cytokines with distinct target cell discrimination (Baggiolini and Dahinden, 1994). Oppenheim et al. (1991) described chemokines as a subgroup of regulatory molecules that have molecular weights ranging from 7 to 15 κD. These similarly-structured proteins function fundamentally as stimulators and chemoattractants for subsets of leukocytes. The recruitment of specific subsets of leukocytes by chemokines induces the stimulation of host defence systems and activation of wound healing. Furthermore, the presence of the variety of leukocytes observed in physiological and pathophysiological states may be due to chemokine expression (Graves and Jiang, 1995). Mitchell and Cotran (2003) described inflamed tissue as being acted upon by

primary proinflammatory mediators (e.g. IL-1 and TNF-α) which in turn stimulate secondary proinflammatory mediators such as chemokines to induce the recruitment and accumulation of neutrophils vs. lymphocytes vs. eosinophils to the affected tissue (Baggiolini and Dahinden, 1994).

Types of Chemokines

Of the 50 known human chemokines, there are four subfamilies of structurally related chemokines which are differentiated from each other according to their arrangement of four specific cysteine domains (C) and amino acid residues (X) (Mitchell and Kumar, 2003; Charo and Ransohoff, 2006). Structurally, the C-X-C subfamily of chemokines has an amino acid residue which separates the first two cysteine residues in the molecule, whereas the C-C chemokines have a structural arrangement in which the first two cysteine residues are adjacent to one another (Adams and Lloyd, 1997). There is a third subfamily of chemokines referred to as lymphotactin, which is deficient in two of the four-cysteine residues present in the C-X-C and C-C subfamilies. Finally, the fourth and most recently discovered chemokine is fractalkine which has three amino acid residues interposed among the first two cysteine domains, and it is designated $CX_3CL$-1. The structural differences of the chemokines are essential to their ability to recruit specific leukocyte subsets (Kelner et al., 1994).

*C-C Chemokines*

The human genes on chromosome 17 and mouse genes on chromosome 11 encode the C-C subfamily of chemokines, also known as β-chemokines (Kelner, et al., 1994). These chemokines primarily activate monocytes. They also stimulate basophils, eosinophils, T-lymphocytes and natural killer cells (NK) (Graves and Jiang, 1995). The main C-C chemokines are monocyte chemoattractant protein-1 (MCP-1; CCL2),

monocyte inflammatory protein-1 alpha (MIP-1α; CCL3), monocyte inflammatory protein-1 beta (MIP-1β; CCL4), and the fourth, called Regulated on Activation Normal T Cell Expressed and Secreted (RANTES; CCL5) (Mitchell and Kumar, 2003; Charo and Ransohoff, 2006). The most completely characterized C-C chemokine is MCP-1. MCP-1 stimulates chemotaxis of monocytes, monocyte release of other cytokines, $Ca^{++}$ flux and expression of integrins, and at elevated levels, MCP-1 causes the generation of oxygen free radicals (respiratory burst) (Valente et al., 1991; Rollins et al., 1991; Jiang et al., 1992). Baggiolini and Dahinden (1994) suggested that MCP-1 is a significant mediator of allergic inflammation due to its ability to induce histamine release from basophils (Kuna et al., 1992). Furthermore, MCP-1 expression has been observed in macrophages/foam cells and smooth muscle cells in atherosclerotic tissue (Yla-Herttuala et al., 1991; Cushing and Fogelman, 1992; Yu et al., 1992).

*C-X-C Chemokines*

The C-X-C subfamily of chemokines, also referred to as α-chemokines, is encoded by the human genes on chromosome 4 and mouse genes on chromosome 5 (Luster and Ravetch, 1987; Mukaida et al., 1989; Kelner et al., 1994). Activated macrophages and tissue cells such as endothelium produce C-X-C chemokines (Mitchell and Kumar, 2003). The discovery of the primary C-X-C chemokine IL-8 (CXCL-8) in 1987, also referred to as neutrophil-activating protein-1 (NAP-1), revealed a small group of chemokines and their prospective functions as chemical mediators (Baggiolini and Dehinden, 1994; Charo and Ransohoff, 2006). Monocytes are the primary producers of IL-8. However, there are various other cells that can be stimulated to produce IL-8, such as endothelial cells, chondrocytes, epithelial cells and several tumor cells. Other proinflammatory agents can cause the generation of IL-8, such as lipopolysaccharides

(LPS), IL-1, TNF-α, bacteria (Barnes et al., 1992), viruses (Becker et al., 1991), immune complexes (Seitz et al., 1991) and integrins (Kasahara et al., 1991). In addition, IL-8 has receptors (binding sites) for the transcription protein nuclear factor kappa B (NF-κB) and nuclear factor IL-6 (NFIL-6) (Mukaida et al., 1989). IL-8 functions to activate neutrophils, but it may also stimulate different subsets of leukocytes (Clark-Lewis et al., 1991; Hebert et al., 1991). Furthermore, IL-8 also induces cytoskeletal modifications, affects intracellular $Ca^{++}$ concentrations, stimulates integrins, generates OFRs, induces neutrophil degranulation and can orchestrate the recruitment of activated monocytes to vascular lesions (Baggiolini et al., 1989; Detmers et al., 1990; Moser et al., 1990; Paccaud et al., 1990; Charo and Ransohoff, 2006). CXCCL-16 is a CXC chemokine, but its functions and structure are similar to a $CX_3C$ chemokine in that its functional domain is attached to a mucin-like stalk tethered to the cell surface (Charo and Ransohoff, 2006). In addition, it mediates adhesion and acts as a soluble chemoattractant once cleaved from its mucin stalk (Charo and Ransohoff, 2006). It is expressed on macrophages and dendritic cells and has been implicated in the genesis of atherosclerosis due to its scavenger receptor affinity for oxidized lipids (Shimaoka et al., 2000).

*C-$X_3$-C Chemokines*

The most recently discovered (1997) chemokine, termed fractalkine ($CX_3CL$-1), is a protein containing 373 amino acids and is the only known member in this subfamily. The structure of this molecule is a $CX_3C$ pleated chemokine joined to a mucin-like framework tethered to the surface of the cell membrane (Bazan et al., 1997). Northern blotting of human pulmonary aortic endothelial cells (HPAEC) and human umbilical

vein endothelial cells (HUVEC) has suggested that $CX_3C$ expression increases after induction by TNF-α or IL-1. In addition, $CX_3C$ chemokines are be surface-bound transmembrane proteins that can be induced by proinflammatory cytokines. Monocytes and lymphocytes express the chemokine receptor $CX_3CR$ and adhere to fractalkine with a high affinity. Bazan et al. (1997) suggested that under physiologic flow conditions, $CX_3C$ chemokines regulate the interphase period of leukocyte trafficking by arresting leukocytes with its extended stalk, to enhance rolling and promote firm adhesion to the luminal surface of the endothelium lining blood vessels (Haskell et al., 1999). The two forms of fractalkine are anchored and soluble. The anchored $CX_3C$ form is chemoattractant for leukocytes, whereas the soluble form is chemoattractant for monocytes and T cells. TNF–α-converting enzyme is responsible for the cleavage of $CX_3C$ from its mucin stalk, and this allows it to circulate and function as a soluble chemoattractant (Charo and Ransohoff, 2006).

*Lymphotactin*

Another recently discovered chemokine (1994) contains only one of the two cysteine residues present in the C-X-C and C-C subfamilies (Charo and Ransohoff, 2006). The terminal genes located on mouse chromosome 1 encode for lymphotactin. Chemotactic studies conducted by Kelner et al. (1994) suggest that lymphotactin is distinct among chemokines due to its ability to stimulate significant intracellular $Ca^{++}$ flux and chemotaxis only in lymphocytes. The production of lymphotactin results from various sources such as activated CD8+ T cells, CD4-CD8-T cell receptor alpha beta, and thymocytes (Kelner et al., 1994).

#### 1.2.4.2.2 Chemokines, Receptor Binding and Signalling in Atherosclerosis

The exact mechanism of action of chemokines and how they direct the activation and movement of cell types is not well understood. However, Charo and Ransohoff (2006) described the amino terminal (Glu-Leu-Arg) as one of the critical sites for binding of the chemokine to the complementary surface G-protein-coupled receptors on target cells. The loop region of the chemokine follows the second cysteine domain and also has significant importance in binding. This region is formed by three-dimensional folding of the chemokine upon itself through the utilization of disulfide bonds linking cysteine residues in the first and second extracellular loops (Graves and Jiang, 1995). According to Baggiolini (2001), the chemokine receptor is a seven transmembrane domain G-protein which spans the endothelium and is coupled to guanosine triphosphate (GTP)-binding proteins. The GTP binding proteins distinguish the first binding site (docking domain) within the loop region. This coupling complex diminishes the movement of the chemokine and allows the correct alignment of the amino terminal. Upon alignment, the triggering domain functions to activate the receptor, thereby inducing signalling discharges of the GTP binding proteins. Thelen et al. (1988) elaborated on the mechanism of chemokine signalling by examining human neutrophils induced by CXCL-8 (IL-8). They demonstrated that pre-treatment of the cells with pertussis toxin blocked chemokine function, pointing out that the receptor was coupled to $G_1$ type GTP-binding protein. It is now understood that all chemokine receptors consist of seven transmembrane domains coupled to G-proteins (Murphy, 1994), and upon stimulation they activate a signalling pathway that results in cellular conformational changes due to rearrangement of actin (Bazan et al., 1997).

### 1.2.5 Tumor Necrosis Factor- alpha and Interleukin-1

The mediation of inflammation involves the proinflammatory cytokines TNF and IL-1. In the process of inflammation, activated macrophages produce TNF-α and IL-1, whereas activated T cells primarily produce TNF-β (lymphokine cytokine) (Dinarello, 1996). Interleukin-1 and its proinflammatory characteristics are related to the development of atherosclerotic lesions, tissue destruction subsequent to ischemia, restenosis after percutaneous coronary intervention, and myocardial dysfunction (Dinarello, 1996). Moser et al. (1989) observed the ability of IL-1 and TNF-α to induce the transendothelial migration of neutrophils through junctional gaps in cultured human endothelial cells. As outlined by Dinarello (1996), the synthesis of IL-1 from lipid-filled foam cells can augment the surface expression of endothelial adhesion molecules that enhance the affinity of inflammatory and lymphocytic cells to the injured endothelium. The presence of IL-1 in atherosclerotic lesions results from infiltrating foam cells that produce IL-1 in reaction to the presence of oxidized low-density lipoprotein (OxLDL) (Thomas et al., 1994). IL-1 causes the production of IL-8 in response to inflammation, which in turn increases the recruitment of neutrophils to the site of injury, with additional local tissue destruction (Porat et al., 1992). Consequently, the resultant myocardial damage correlates with neutrophil infiltration. Moreover, dogs with induced ischemia from coronary occlusion that were administered IL-1Ra which blocks the receptor of IL-1, experienced attenuated neutrophilic infiltration and myocardial infarct size (Karolle et al., 1991). Not only does IL-1 have deleterious effects on the coronary arteries, it also affects myocardial function. Roberts et al. (1992) revealed that the innate rhythmic beating of cultured neonatal rat myocytes is abolished by the administration of inhibitory IL-1β and transforming growth factor beta (TFG-β). Furthermore, IL-1β and

IL-1R1 gene production was found in endomyocardial biopsies from subjects with myocarditis and dilated cardiomyopathies (Han et al., 1991).

1.2.6 Tumor Necrosis Factor-Alpha (TNF-α)

TNF-α is a cytokine that functions as an immune regulator of inflammation. It has also been suggested that TNF-α is involved in the chemotactic response of activated monocytes or macrophages, T and B lymphocytes, mast cells, smooth muscle cells and fibroblasts at sites of vascular injury, particularly the arterial wall where atheromatous lesions are formed (Furie and McHugh, 1989; Postlewaite and Seyers, 1990; Raines and Ross, 1993; LeBoeuf and Schreyer, 1998).

Significant quantities of TNF-α have not been found in normal vasculature. However, it has been shown to be associated with intimal vascular smooth muscle cells (VSMCs) within plaques of atherosclerotic arteries. It is also found in the arterial walls of animal models of transplantation-associated atherosclerosis (Baranth et al. 1990; Rayment et al., 1996; Tanaka et al., 1995b). Moreover, in an animal model of coronary-graft atherosclerosis, Clausell et al. (1994) demonstrated that obstruction of TNF-α with a soluble TNF-α receptor inhibited coronary neointimal development. Consequently, they concluded that the cells of the arterial wall produced TNF-α. In the injured vasculature, TNF-α may play a role in controlling the production of growth factors such as PDGF, vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), adhesion molecules, other cytokines, extracellular matrix degrading metalloproteinases (MMPs) and the growth and migration of VSMC (Winkles and Gay, 1991: Yoshida et al., 1997; Couffinhal et al., 1993; Loppnow et al., 1989; Galis et al., 1995).

TNF-α exists in two forms: the common 17 kD soluble form and the membrane-bound form, both of which stimulate biological activities. The synthesis of TNF-α comprises

the production of a 26 kD precursor of TNF-α that occurs as a cell surface type II transmembrane protein (Kriegler et al., 1988). This transmembrane protein precursor is proteolytically cleaved by TNF-α–converting enzyme (TACE) to generate trimers of the soluble form of TNF-α (sTNF-α) (Decoster et al., 1995; Georgopoulos et al., 1996). The modulation of TACE influences the plasma concentrations of membrane and diffusible forms of TNF-α, and thus controls the paracrine and endocrine effects of TNF-α (LeBoeuf and Schreyer, 1998).

1.2.6.1 TNF-α Receptors

Cell surface receptors existing on arterial endothelium and on cells involved in atheromatous lesion development are involved in the mediation of the TNF-α response. The two cell surface receptors for TNF-α are TNFR1 (p55) and TNFR2 (p75). The variation in the surging signals generated by these receptors is responsible for the pleiotrophic effects induced by TNF-α (LeBoeuf and Schreyer, 1998). Some of the biological developments resulting from binding of TNF-α to p55/p75 that may be related to the genesis of atherosclerotic lesions are as follows: NF-κB activation (Pfeffer et al., 1993); adhesion molecule induction (Neumann et al., 1996); a decrease in endothelial constitutive nitric oxide synthetase production (Marczin et al., 1996); an increase in endothelial macrophage colony stimulating-factor (M-CSF) (Clinton et al., 1992); an increase in the effect of endothelial tissue factor (Paleolog et al., 1994); an increase in monocyte and or macrophage differentiation (Libby and Galis, 1995); a decrease in monocyte or macrophage Apo E activity (Zuckerman and O'Neal, 1994); an increase in monocyte or macrophage superoxide production (Tanner et al., 1992); an increase in monocyte or macrophage nitric oxide production (Miller et al., 1996); inhibition of insulin signalling (Peraldi et al., 1996); an increase in smooth muscle cell proliferation

and migration (Tanaka et al., 1996; Jovinge et al., 1997); and an increase in smooth muscle cell expression of adhesion molecules and production of M-CSF (Braun et al., 1995; Clinton et al., 1992). On the other hand, the anti-atherosclerotic effects resulting from binding of TNF-α to p55/p75 (antagonistic effects of cytokines as described earlier) are as follows: a decrease in monocyte or macrophage lipoprotein lipase activity (Tengku-Mohammed et al., 1996); an increase in monocyte or macrophage Apo E activity (Duan et al., 1995); and finally, a decrease in monocyte or macrophage scavenger receptors for mRNA (Hsu et al., 1996). Since TNF-α elicits a cascade of chemokines that have both proinflammatory and anti-inflammatory actions, it is possible that the presence or absence of p55/p75 receptors creates an imbalance that helps to determine whether TNF-α functions as a protective or damaging cytokine in relationship to atherosclerosis (LeBoeuf and Schreyer, 1998).

1.2.6.2 TNF-α and Vascular Permeability

One of the primary functions of the endothelium is to act as a cellular enclosure restricting blood cells within the extracellular fluid compartment. Thus, under physiologic conditions vascular permeability is tightly regulated by the endothelium (Simionescu, 1988). Brett et al. (1989) reported that macrophage-derived TNF-α may have the ability to alter membrane permeability, induce vascular leakage by rearrangement of cytoskeletons, stimulate the conformational change of cell shape and increase the formation of intercellular gaps. In animal models, administration of TNF-α was found to exhibit similar toxic characteristics such as vascular leakage and impaired endothelial barrier functions comparable to those animals that were infused with Escherichia coli/LPS (Tracy et al., 1986). Pertussis toxin inhibits G-proteins from interacting with G-protein-coupled receptors on the cell membrane, thus impeding

intracellular communication. The discovery that pertussis toxin administered to cultured bovine aortic endothelial cells inhibited the cellular conformational changes, cytoskeletal rearrangement and increases in vascular permeability led to the proposed existence of a receptor. The TNF-α-dependent signal transduction pathway is central to endothelial cells associated with a pertussis toxin-sensitive regulatory G-protein (Brett et al., 1989). Thus, the pertussis toxin-sensitive G-protein may be responsible for the endothelial barrier function. Furthermore, it is speculated that TNF-α stimulates an increased and significant change in the actin-based cytoskeletal alteration associated with formation of intercellular gaps central to vascular permeability (Brett et al., 1989).

1.2.7 Activated Endothelium

The development of diseases such as atherosclerosis is associated with endothelial activation, notably the endothelial-leukocyte adhesion cascade. The vascular endothelium functions in inflammatory leukocyte selection through the formulation of adhesion molecules and chemoattractant cytokines. Under physiologic and pathophysiological flow conditions, the vascular endothelial lining communicates with peripheral circulating leukocytes (Luscinskas and Gimbrone, 1996). However, during inflammatory conditions, the endothelium expresses discrete vascular endothelial adhesion molecules which attach to their complementary leukocyte counterreceptor/ligands under the influence of proinflammatory cytokines (IL-1 and TNF-α), LPS and specific gram-negative bacterial endotoxins (Bevilacqua et al., 1985; Schleimer and Rutledge, 1986).

Luscinskas and Gimbrone (1996) detailed the adhesion cascade in which cellular and molecular events are involved in the transudation of leukocytes from the microcirculation to locations of inflammation. The attachment of leukocytes to their

complementary endothelial receptor ligands results in rolling of the leukocyte followed by solid bonding (arrest/spreading) to the endothelium. This is followed by transmigration of the leukocytes through the endothelial gaps into the extravascular zone.

1.2.7.1 Adhesion Molecules

The four gene families that mediate the attachment of vascular endothelial adhesion molecules, also referred to as endothelial leukocyte adhesion molecules or ELAMs, to their analogous leukocyte counterreceptors are as follows: (1) the selectins, of which there are three types: (a) E-selectin (originally identified on endothelial cells); (b) P-selectin (originally identified on platelets); and (c) L-selectins (originally identified on lymphocytes); (2) the three immunoglobulin's, as follows: (a) intercellular adhesion molecules (ICAM-1, -2, -3); (b) vascular adhesion molecule-1 (VCAM-1); and (c) platelet-endothelial cell adhesion molecule-1 (PECAM-1) also known as CD31; (3) mucin-like adhesion molecules [P-selectin glycoprotein ligand (PSGL-1)]; and (4) the integrins β-1 and β-2 (Springer, 1994; Carlos and Harlan, 1994; Bevilacqua and Nelson, 1993).

1.2.7.1.1 Selectin Adhesion Molecules

As described by Bevilacqua and Nelson (1993), the name selectin was fundamentally recommended to emphasize the existence of the lectin domain on the molecule as well as to feature the molecule's selective properties of expression and action. To establish an adhesive relationship between leukocytes, platelets and endothelial cells, the three selectins function in agreement with other cell adhesion molecules (i.e. ICAM-1, VCAM-1 and integrins) (Luscinskas and Gimbrone, 1996). Specifically, selectins regulate the binding of flowing leukocytes to the vessel wall and allow leukocytes to roll

(in milliseconds) in the direction of flow (Lawrence and Springer, 1991). The selectins function as a calcium-controlled lectin expressed on the leukocyte which recognizes and attaches to a heterogeneous class of glycoproteins (counterreceptors) that extend tethered oligosaccharides (ligands) for their adherence to vascular endothelium and platelets (Luscinskas and Gimbrone, 1996). The controlled presence of selectins and their counterreceptors (ligands) serves to start and end the inflammatory response. Thus, untimely expression of these adhesion molecules may be instrumental in leukocyte-mediated tissue destruction, especially in atherosclerosis (Lawrence and Springer, 1991).

The selectins (E-, P- and L-) express characteristic structural distinctiveness because of the variety and configuration of their domains. The extracellular region of each selectin molecule consists of an amino ($NH_2$) terminal domain parallel to C-type lectins and contiguous with an epidermal growth factor (EGF)-like domain. There are fluctuating numbers of regulatory-like modules (60 amino acids in length with 6 cysteinyl residues) alongside a transmembrane sequence followed by a cytoplasmic array at the carboxyl end of each selectin (Lasky et al., 1989) which facilitate the adhesion of leukocytes to the vessel wall.

*E-Selectin*

Bevilacqua et al. (1987) specified E-selectin (CD62E) (ELAM-1) as a 115 κD antigen that was stimulated on HUVEC subsequent to induction by IL-1. The molecular cloning of E-selectin by Hession et al. (1990) revealed the essential C-type lectin-binding domain, the crucial EGF-like binding domain and six regulatory protein zones. The main counterreceptors for E-selectins are carbohydrate ligands such as sialyl Lewis$^x$ and

the isomer sialyl Lewis$^{a}$ (sLe$^{x}$, sLe$^{a}$) or analogous configurations (Tiemeyer et al., 1991; Springer, 1994).

The unusual expression of E-selectin on the endothelium of vascular beds allows it to function as a marker for vascular tissue endothelial cell activation (Luscinskas and Gimbrone, 1996). Following cytokine (IL-1 and TNF-α) or LPS activation, E-selectin is expressed on activated endothelium and stimulates the adhesion of neutrophils, monocytes, some T-memory lymphocytes, eosinophils and basophils (Springer, 1994; Bochner et al., 1991).

*P-Selectin*

Bevilacqua and Nelson (1993) identified P-selectin (CD62) as a cell surface receptor protein of approximately 140 κD that is synthesized and stored within cytoplasmic endothelial storage granules, termed Weibel-Palade bodies, and in alpha-granules in dormant platelets. P-selectin is quickly dispersed to the cell surface to function as an adhesion molecule upon platelet and endothelial activation by secretegogues (i.e. histamine, thrombin), proinflammatory cytokines (IL-1, and TNF), LPS, and $H_2O_2$ to bind neutrophils and monocytes (Luscinskas and Gimbrone, 1996; Springer, 1994; Patel et al., 1991). However, the exposure of P-selectin to activated platelets and endothelium is short and decreases within minutes (Bevilacqua and Nelson, 1993).

Although P-selectin is expressed on stimulated platelets and endothelium, it has been known to bind to ligands on leukocytes (McEver and Cummings, 1997). P-selectin binds with high affinity to counterreceptor carbohydrate ligands (sLe$^{x}$, sLe$^{a}$) and a limited amount of cell surface glycoprotein (Moore et al., 1991; Larsen et al., 1992). Studies conducted by Moore et al. (1991) demonstrated that P-selectin platelet-neutrophil

binding was terminated by the administration of proteases, implying that the ligands (glycans) on neutrophils are glycoproteins. The number of binding regions (10,000-20,000/cell) for platelet P-selectin on neutrophils comprises a limited fraction of the total of its cell surface, suggesting that an ideal configuration of P-selectin with its glycoprotein counterreceptor must be achieved to allow for sufficient adhesion (Moore et al., 1991; 1992). In addition, ligand blotting and affinity chromatography studies conducted by Moore et al. (1991) suggest that P-selectin binds exclusively to a single glycoprotein in human myeloid cells. This molecule is now called P-selectin glycoprotein ligand-1 (PSGL-1), and it functions to increase adhesiveness of both P- and L- selectin to leukocytes in areas of inflammatory and thrombotic pathogenesis (McEver and Cummings, 1997; McEver, 2002).

*L-Selectin*

L-selectin (CD62L) is present on all circulating leukocytes and in a subpopulation of memory lymphocytes (Carlos and Harlan, 1994; Springer, 1994). Molecular cloning of L-selectin has demonstrated its mechanism of adhesion to the activated lymphoid and non-lymphoid murine endothelium and involvement in leukocyte rolling on the vessel wall before firm attachment. There is 80% homology between human and murine L-selectin with preservation of lectin, EGF, transmembrane and cytoplasmic regions (Robinson et al., 1998). L-selectin has also been reported to bind to the $sLe^x$ or associated fucosylated structures of P-selectin. Finally, L-selectin binds to the following: (1) unknown cytokine-induced counter structures expressed on non-lymphoid endothelium; and (2) sialylated, fucosylated, and sulphated carbohydrate complexes expressed on murine glycosylation-dependent cell adhesion molecules (GlyCAM-1) (Carlos and Harlan, 1994).

1.2.7.1.2 Integrin Adhesion Molecules

Integrins are primary receptors which function to attach cells to the extracellular matrix, moderate significant cell-cell adhesion reactions, and are involved in cell signal transduction. Many human diseases interfere with integrin activity, thus the use of antibodies or peptides present several possibilities for therapeutic usage in thrombosis, cancer and atherosclerosis (Hynes, 1992). Integrins are mononuclear leukocyte receptors that comprise a huge family of transmembrane heterodymeric glycoproteins. All integrins contain non-covalently bound α and β subunits that range in size between 120 and 180 κD and 90 to 110 κD, respectively. Either α or β receptors can identify one or more extracellular ligands or counterreceptors on other cells (Hynes, 1992). All of the known $\beta_1$ (CD29) and $\beta_2$ (CD18) integrin subfamilies are articulated on monocytes and mediate endothelial adhesion via their counterreceptor intercellular adhesion molecules (ICAM-1, -2, and -3). The $\alpha 4\beta_1$-integrin, however, also known as very late antigen-4 (VLA-4), is dually expressed on lymphocytes and monocytes and functions as a ligand for the endothelial-articulated counterreceptors VCAM-1 and fibronectin (Elices et al., 1990). The VLA-4/VCAM pathway has been demonstrated to mediate leukocyte rolling and orchestrate arrest and transmigration of lymphocytes and monocytes under flow conditions *in vitro* (Luscinskas and Gimbrone, 1996). However, the VLA-4/fibronectin binding occurs by a mechanism that is independent from the VLA-4/ VCAM pathway (Elices et al., 1990). The $\beta_2$ subunit can combine with any of the three α subunits, αL (CD11a, LFA-1), αMβ2 (CD11b) and αX (CD11c). Lymphocyte subsets express only CD11a/CD18, whereas monocytes express all three heterodimers on their plasma membrane (Diamond and Springer, 1993). Additionally, activated monocytes have a reservoir of CD11b/CD/18 and CD11c/CD18 molecules

stored in secretory granules that can be quickly released to the surface for enhanced endothelial adhesion. Complement binding, phagocytosis and adhesion to extracellular matrix proteins are some of the salient features of $\beta_2$ integrins (Luscinskas and Gimbrone, 1996). $\beta_2$ integrins also have an important role in host defence as demonstrated in the hereditary disease endothelium leukocyte adhesion deficiency syndrome (LAD). Patients with LAD have nonexistent or impaired $\beta_2$ integrins on their leukocytes which causes adhesion and mobility irregularities, with symptoms of recurrent bacterial infections and decreased pus at areas of inflammation (Hynes, 1992).

1.2.7.1.3 Immunoglobulin Adhesion Molecules

Numerous immunoglobulin superfamily (IgSF) adhesion molecules, which are expressed on the endothelium, bind to their counterreceptors (integrins) that are expressed on circulating leukocytes. The degree of IgSF adhesion molecule expression is augmented by proinflammatory mediators, particularly proinflammatory cytokines (Luscinskas and Gimbrone, 1996). As products of specific and homologous genes, ICAMs-1, 2, and 3 were all originally discovered by their capacity to bind to the integrin, lymphocyte function associated antigen-1 (LFA-1) ($\alpha L\beta 2$) on neutrophils and monocytes under the influence of IL-8 (Springer, 1990). Likewise, ICAM-1 can also adhere to the integrin MAC-1 ($\alpha M\beta 2$) on neutrophils and monocytes under the influence of IL-8 (Diamond et al., 1991). Lipoprotein(a) can also increase the expression of ICAM-1 in cultered human endothelial cells (Takami et al., 1998). Furthermore, ICAM-1 cytokine-induced expression on the endothelium may influence cell-cell interactions and leukocyte transmigration at sites of inflammation (Springer, 1994). Similarly, ICAM-2 is expressed on the endothelium as well. Cytokines or LPS do not control its expression, but they may mediate leukocyte influx in uninflammed tissue similar to

lymphocyte recirculation (Springer, 1994). Platelet endothelial cell adhesion molecule-1 (PECAM-1) is a member of the immunoglobulin super family of adhesion molecules that is expressed on endothelium, monocytes and specific lymphocytes (Springer, 1994).

### 1.2.8 Vascular Cell Adhesion Molecule-1 (VCAM-1) and Atherosclerosis

During the inflammatory response, the adhesion molecule receptors on the activated endothelial surface signal to counterreceptors on circulating leukocytes, resulting in their arrest, spreading and subsequent transmigration into the subendothelium. The initial action in the commencement of atherosclerosis is the attachment of mononuclear cells to the vascular endothelium (Gerrity, 1981; Faggiotto et al., 1990; Ross, 1986). VCAM-1, an inducible endothelial surface protein, has been shown to facilitate endothelial adhesion of monocytes and T-lymphocytes (Springer, 1990). Since monocytes and T-lymphocytes are the main participants in the development of the atherosclerotic lesion, it is thought that VCAM-1 plays a central role in the recruitment of inflammatory cells into the progressing atherosclerotic plaque. The substantiation of this association arises from animal studies on Watanabe hyperlipidemic rabbits that demonstrated through immunoreactivity that VCAM-1 was present on arterial endothelium overlying fatty streaks (Cybulsky et al., 2004). O'Brien et al. (1993), while investigating the function of endothelial VCAM-1 expression, gathered similar evidence from human coronary atherosclerotic plaques by performing immunocytochemical studies using rabbit polyclonal antisera raised against recombinant human VCAM-1. The investigators established the existence of VCAM-1 connected to the intimal neovasculature layer located at the base of human coronary atherosclerotic lesions. This evidence supports the notion that VCAM-1 functions in the recruitment of leukocytes into atheromatous plaques.

1.2.8.1 VCAM-1 Specificity

The specificity of endothelial cells which delineate the types of leukocytes recruited, such as monocytes, lymphocytes or neutrophils, is determined by which adhesion molecules (i.e. VCAM-1, ICAM-1 and E-selectin) are expressed as a result of the inflammatory stimuli (Elices et al., 1990). For example, VCAM-1 has been linked with the early stages of diabetic and non-diabetic atherosclerosis. The cytokine-induced expression of VCAM-1 on the activated endothelial surface interacting with its integrin counterreceptor, VLA-4, on circulating monocytes provides a pathway for monocytes to bind to the vessel surface leading to the genesis of the atherosclerotic lesion (Schmidt et al., 1995a). However, the type of inflammatory signals and related molecular mechanisms that stimulate the expression of VCAM-1 in endothelial cells in the genesis of the atherosclerotic lesion remain an enigma. For instance, chemical mediators frequently associated with atherogenic lesions, such as TNF-α and IL-1β, induce the expression of not only VCAM-1, but also ICAM-1 and E-selectin in cultured endothelial cells. Thornhill et al. (1990) have demonstrated that IL-4 increases TNF-α stimulation of endothelial VCAM-1 expression. However, it inhibits TNF-α activation of ICAM-1 and E-selectin expression. Assuredly, there are various other regulatory signals that may induce a VCAM-1 distinctive controlling process to either stimulate or inhibit VCAM-1 expression apart from ICAM-1 or E-selectin expression. Recent studies focusing on the pathogenesis of atherosclerosis used HUVEC to identify oxidative stress generation as a signalling pathway resulting in VCAM-1 gene expression. Specifically, reactive oxygen species (ROS) function in regulating VCAM-1 expression through mediation of NF-κB activation (a transcription regulatory protein) (Schreck et al., 1992a; Pahl and Baeuerle, 1996). Conversely, oxidative stress does not appear to be necessary in the activation of

ICAM-1 or E-selectin in HUVEC through NF-κB (Marui et al., 1993). A more recent study conducted by Tummala et al. (2000) investigated the role of oxidation-reduction-coupled reactions in the modulation of expression of VCAM-1 in atherosclerotic lesions of human aortic endothelial cells (HAEC) and human microvascular endothelial cells (HMEC). These researchers concluded that flavin-binding proteins such as nicotinamide adenine dinucleotide dehydrogenase (NADH)/reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase may control VCAM-1 gene expression separately from NFκB. In addition, they suggested that superoxide ($O_2^-$) production is not necessary for NFκB activation. Expressed differently, the data from this study suggest that cytokines could induce oxidative stress by elevating $O_2^-$ generation. However, this increase may not function as a second messenger in NF-κB activation in endothelial cells. Nevertheless, there is a considerable body of evidence that supports a role for ROS in the TNF-α induced expression of VCAM-1 in the early stages of development of the atherosclerotic lesion.

1.2.8.2 Soluble VCAM-1 (sVCAM-1)

The adhesion of circulating leukocytes and monocytes to endothelial cells and consequent transmigration are crucial steps in the genesis and progression of atherosclerotic lesions (Hillis and Flapman, 1998; Jang et al., 1994). VCAM-1 plays an important role in SMC migration by joining with the actin cytoskeleton and the extracellular matrix (Moiseeva, 2001). VCAM-1 expression in smooth muscle cells in regions susceptible to the development of atherosclerosis before mononuclear cell infiltration implies its functional role in atherosclerotic lesion development. VCAM-1 may also be instrumental in SMC migration and proliferation in neointimal formation after mechanical injury from percutaneous coronary intervention (PCI). PCI is an

invasive interventional procedure utilizing a balloon-tipped catheter system and/or stents to diminish the ischemic effects of coronary artery disease. In studies on cultured endothelial cells, it has been shown that interactions of VCAM-1 are necessary for phenotypic alterations producing a more contractile cell type. This implies that VCAM-1 may influence the cellular composition of the lesion and plaque stability (Braun et al., 1999; Lee and Libby, 1997).

sVCAM-1 is the soluble form of VCAM-1 and its plasma levels indirectly reflect the concentration of endothelial VCAM-1 expressed on the cell surface. The circulating form of VCAM-1 is produced by proteolytic cleavage from the cell surface, and the concentration of soluble molecules reflects increased expression of cell adhesion molecules on endothelial cells (Pigott et al., 1992). Miwa et al. (1997) identified increased levels of soluble adhesion molecules in patients with variant angina, and these were associated with increased risk of future MI (Ridker et al., 1998b). Postadzhiyan et al. (2008) demonstrated that in patients with acute coronary syndrome, elevated serum sVCAM-1 concentrations positively correlated with an increased risk for future cardiovascular events. This suggests that sVCAM-1 may enhance the information acquired from conventional biochemical markers.

1.2.8.3 VCAM-1, Cytokines and Mevalonate Synthesis

The initiation and development of atherosclerotic plaques requires the expression of vascular endothelial adhesion molecules such as VCAM-1 and E-selectin, and their subsequent attachment to ligands on circulating leukocytes, thus leading the way to their transmigration into the vascular wall. Proinflammatory cytokines such as TNF-α and IL-1β can stimulate the expression of VCAM-1 and E-selectin in endothelial cells throughout the development of atherosclerotic lesions (Wang and Springer, 1998).

Interestingly, a relationship has been identified between the cytokine-induced expression of VCAM-1 and the synthesis of cholesterol. In order to describe this relationship, one must understand the mevalonate pathway that is involved in the synthesis of isoprenoids that control cholesterol production and cell growth. In part, the biosynthetic mevalonate pathway regulates the synthesis of cholesterol from two sources: (1) endogenous (internal), by synthesis from acetyl coenzyme A (acetyl CoA) through mevalonate; and (2) exogenous (external), from the receptor-induced uptake of plasma low-density lipoprotein (LDL) (Goldstein and Brown, 1990). The prevention of pathophysiological increases in cholesterol is achieved through a negative feedback mechanism which creates a balance between the internal and the external sources of cholesterol production. The production of mevalonate is involves two enzymes: (1) 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) synthetase, which converts acetyl CoA + acetoacetyl CoA to HMG-CoA; and (2) 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) reductase, which converts HMG-CoA to mevalonate. Mevalonate proceeds further to produce sterols: cholesterol (a major structural component in cell membranes, vitamin D, steroid hormones, bile acids and lipoproteins), ubiquinone (takes part in electron transport) and dolichol (required for glycoprotein synthesis). The blockade of mevalonate synthesis inhibits the formation of the preceding products and inhibits cell proliferation and growth (Beck et al., 1988; Wolda and Glomset, 1988; Glomset et al., 1990). Recently, therapeutically administered cholesterol synthesis blocking agents (vastatins/statins) for decreasing plasma LDL have been developed which function primarily to inhibit HMG-CoA reductase (Kinlay et al., 1996; Luscher et al., 1996; Maron et al., 2000). Vastatins such as mevastatin, simvastatin and pravastatin can also exhibit pleiotropic effects such as: (1) anti-atherothrombotic functions; (2) the control of arterial myocyte and vascular

SMC proliferation; (3) decrease in the generation of ROS from monocytes/macrophages; and (4) increase in the level of nitric oxide synthetase and reduction in the production of endothelin from endothelial cells, thereby altering vascular wall activity connected to the genesis of atherosclerosis (Rosenson and Tangney, 1998; Vaughan et al., 1996; Corsini et al., 1996; Giroux et al., 1993; Laufs et al., 1998; Hernandez-Perera et al., 1998; Veillard and Mach, 2002). Rasmussen et al. (2001), using human cultured endothelial cells from collagenase-digested umbilical veins, investigated whether statins (inhibition of HMG-CoA reductase with 0.1-1μM of mevastatin) could regulate the TNF-α-induced endothelial surface expression of VCAM-1 and E-selectin. Their findings demonstrated that HMG-CoA reductase inhibition resulted in a decrease in TNF-α-induced endothelial VCAM-1 expression. However, there was an increase in E-selectin expression. These data suggest that TNF-α-induced expression of VCAM-1 may be under the influence of HMG-CoA. Furthermore, the mevalonate pathway may be involved in the TNF-α induced expression of VCAM-1 on the activated endothelium in the initial stages of the development of the atherosclerotic plaque (Rasmussen et al., 2007).

### 1.3 Advanced Glycation End Products

As explained by Gugliucci (2000), Maillard is credited with discovering the glycation reaction in 1912 while examining foods. He observed that sugars react nonenzymatically (glycosylation) with a multitude of proteins to form early glycation products such as Amadori products or fructosamine (Maillard, 1912). The complete reaction involving sugars and proteins in foods produces the brown pigments observed in beer, cola, breads and piecrusts. In humans, any glycated plasma protein in the first stage is known as fructosamine (fructosamino-protein adducts) and the analysis of the glycated form of hemoglobin ($HbA_1$) for the purpose of clinical detection and glycemic

control is referred to as the fructosamine assay (Benjamin and Sacks, 1994). In recent times, glycation has been referred to as the nonenzymatic reaction of sugars with proteins or lipids or nucleic acids to form early glycation products. Through a series of rearrangements and oxidative reactions of early glycation products, the second stage of glycation occurs, producing advanced glycation end products (AGEs) (Hunt et al., 1990).

AGEs were originally identified by their yellow-brown fluorescent color and their potential to form cross-links among proteins/amino acids (Vlassara et al., 1984). The nonenzymatic Maillard reaction is the classical route of AGE formation. The Maillard pathway consists of the reaction between glucose or other reducing sugars and the N-terminal amino acid groups and/or ε-amino acid residues of proteins to produce Schiff base adducts (early glycation products), referred to as aldimines. Successive chemical rearrangement of aldimine causes the generation of reversible Amadori adducts (intermediate glycation products) such as fructose-lysine. Ultimately, Amadori products undergo further chemical rearrangement through a series of dehydration, β-elimination and condensation reactions to form irreversibly cross-linked proteins (advanced glycation end products) (Maillard, 1912; Njorge and Monnier, 1989; Bucala and Vlassara, 1995; Bucala et al., 1995). Similarly, the glycation reaction of glyceraldehydes with protein establishes protein cross-linking to form alpha-hydroxyaldehydes (Acharaya and Manning, 1983). Currently, however, the term "advanced glycation end products" is used to describe a vast domain of AGEs of the Maillard reaction comprising compounds such as 3, 4-N- (carboxymethyl) lysine (CML) and pyrraline, which do not display color, fluorescence, or occur as, cross-links of proteins (Reddy et al., 1995; Hofmann et al., 1999). The synthesis of AGEs *in vitro* and

*in vivo* is contingent upon the turnover rate of the chemically transformed target, time and sugar concentration (Bierhaus et al., 1997a). Interestingly, glucose has the slowest glycation rate of all sugars, whereas intracellular sugars such as fructose, threose, glucose-6-phosphate, and glyceraldehyde-3-phosphate generate AGEs at a more accelerated rate (Bann et al., 1981; Takagi et al., 1995; Suarez et al., 1989).

### 1.3.1 AGEs and VCAM-1

Historically, the excessive increases in AGEs in the vascular wall collagen and basement membrane were a function of age and levels of glycemia (Monnier and Cerami, 1981). Recently, AGEs have been shown to not only have the ability of cross-linking proteins, but also to exhibit an array of biological functions. Under conditions of hyperglycemia, AGEs are increased in circulating plasma and deposited in the vascular walls at sites of vascular tissue injury. The expression of receptors for advanced glycation end products (RAGEs) is also increased at sites of vascular injury, allowing trapping and accumulation of AGEs on subendothelial collagen lining of vascular walls and basement membranes (Bierhaus et al., 2005). The trapping of AGEs in diabetic vasculature (subendothelial wall) induces the expression of both VCAM-1 and its soluble form sVCAM-1, resulting in an increased affinity for circulating monocytes on the activated endothelium. Consequently, monocytes bind to the vessel surface where they transmigrate through the vessel wall and likely augment the progression of atherosclerosis (Schmidt et al., 1995a). Schmidt et al. (1996) later demonstrated that plasma levels of sVCAM-1 are higher in diabetic patients with microalbuminuria as compared to normoalbuminuric patients. Furthermore, their findings suggested that the blockage of the AGE receptor (RAGE) might lower sVCAM-1 levels and possibly serve as a therapeutic mechanism to minimize diabetic vasculopathies. Boulanger et al.

(2002) investigated the AGE-RAGE interaction on adhesion molecule expression and leukocyte binding in peritoneal dialysis (PD) of patients with end-stage renal disease. The most common complications observed in long term PD are sclerosing syndromes and membrane failure due to a loss of mesothelial cells and barrier function. They hypothesized that AGE formation was associated with the elevated glucose concentrations of peritoneal dialysis fluids and that AGEs could change mesothelial cell function by binding to RAGEs on human peritoneal mesothelial cells (HPMC), resulting in the induction of endothelial VCAM-1 expression and leukocyte adhesion. Their findings concluded that the AGE-RAGE interaction increased mesothelial cell activity, and produced an increase in VCAM-1 expression which augmented the local inflammation and peritoneal damage found in long-term PD patients.

Basta et al. (2004) have postulated that AGE-RAGE interaction in endothelial cells activates the intracellular production of ROS that are involved in a signal transduction pathway, and which in turn results in the expression of VCAM-1. Utilizing specific inhibitors (apocynin and diphenylene iodium) for NADPH oxidase and thenoyltrifluoroacetone (TTFA) for mitochondrial electron transport chain, the investigators found a significant decrease in AGE-induced ROS generation and VCAM-1 expression in cultured endothelial cells. The data gathered from their study lend support to previous studies which demonstrated that AGE interaction with RAGE induces ROS which subsequently stimulates the redox-sensitive transcription factor NF-κB to induce the expression of response to injury genes such as VCAM-1, which has been shown to be present in early atherosclerotic lesion formation (Yan et al., 1994; Schmidt et al., 1995a).

### 1.3.2 Receptors for Advanced Glycation End Products (RAGE) and Soluble Receptors for Advanced Glycation End Products (sRAGE)

Stimulation of the multiligand receptors for advanced glycation end products has been suggested in the progression of diabetic and nondiabetic atherosclerosis (Lyons, 1993; Bierhaus et al., 2005; Yamagishi et al., 2006). RAGE, a member of the immunoglobulin superfamiliy of cell surface molecules, is a 45-kD protein originally isolated from bovine lung endothelium based on its ability to bind to AGE ligands (Schmidt et al., 1992). Tan et al. (2006) detailed the classification of three gene splice mRNA variants of human RAGE. The first encodes for the full-length RAGE which consists of extracelluar and transmembrane domains that function in the binding for AGEs and signaling necessary for cellular activation. The extracellular region of full length RAGE is made up of one V-type (variable) immunoglobulin domain and two C-type (constant) domains attached together by internal disulfide bridges interposing the cysteine residues (V-C-C). The transmembrane region contains a short hydrophobic transmembrane domain followed by a cytoplasmic domain (tail) essential for signal transduction (Schmidt et al., 2000). The interaction of RAGE ligands with RAGE induces cellular activation with the deleterious effects of the production of response to injury genes. The second mRNA variant, termed N-truncated RAGE, encodes for a membrane-bound isoform devoid of an AGE-binding domain. Specifically, the N-truncated type is identical to full length RAGE in that it has the two C-type domains (C-C); however, it lacks the V-type domain necessary for binding of RAGE ligands (nonbinding, nonsignaling). The third isoform, called sRAGE, is C-truncated and possesses the same immunoglobulin domains present in full-length RAGE including AGE-binding patterns (V-C-C) but is deficient in the cytosolic and tranmembrane signaling domains. There are

two types of sRAGE both of which are C-truncated and circulating. One form of sRAGE termed esRAGE is produced by alternative splicing of RAGE mRNA and is secreted extracellularly and binds to AGE but has no transmembrane domain to induce signaling and cellular activation. The other form of soluble RAGE circulating in the blood is also devoid of signaling activity, and is proteolytically cleaved from the cell surface by matrix metalloproteinases (MMPs) (Katakami et al., 2008). To date there are only two commercially available enzyme-linked immunoassay (ELISA) kits for the detection of soluble RAGE. They are as follows: (1) the ELISA assay for total soluble (sRAGE) that uses antibodies that detect total circulating soluble RAGE. The total soluble RAGE is the sum of esRAGE and shed RAGE from the cell surface; and (2) the ELISA that detects esRAGE using a polyclonal antibody raised against the characteristic C-terminus of the esRAGE sequence (Katakami et al., 2008). The current study used the ELISA kit that measured total sRAGE.

The normal plasma sRAGE concentration in humans ranges from 1000 to 1500 pg/mL (Falcone et al., 2005). According to a review by Prasad (2006a), the levels of sRAGE are decreased in pathological states such as CAD, metabolic syndrome, diabetes, rheumatoid arthritis, and Alzheimer disease. Interestingly, sRAGE has been expressed and purified from baculovirus-transfected insect cells and by a purification method from the yeast *Pichia pastoris* for use in vivo experiments (Hoffmann et al., 1999; Ostendorp et al., 2006). There is tissue specificity in the expression characteristics of sRAGE (Schleuter et al., 2003). It has been demonstrated that the total circulating concentrations of this isoform are attenuated in diabetic and nondiabetic subjects with coronary artery disease and those with essential hypertension (Falcone et al., 2005; Tan et al., 2006). The data suggest that sRAGE may have a protective effect against AGEs

by blocking their attachment to RAGEs (Bierhaus et al., 2005; Falcone et al., 2005; Tan et al., 2006). Geroldi et al. (2006) implied that sRAGE may be a biomarker of longevity in humans. sRAGE has been reported to decrease plasma concentrations of VCAM-1, tissue factor and RAGE in animal models (Kislinger et al., 2001). The therapeutic use of sRAGE or other RAGE inhibitors (aminoguanidine) may be of future value in controlling the pathophysiological pathways involved in diabetic and non-diabetic CAD (Yamagishi et al., 2008a).

1.3.2.1 Drug Factors Affecting Expression of sRAGE

Angiotensin-converting enzyme inhibitors (ACEi) and angiotensin receptor blockers (ARBs) decrease blood pressure and the accumulation of AGEs in the arterial wall (Yagamishi et al., 2008a; Yagamishi et al., 2008b; Schleicher and Friess, 2007). Furthermore, exposure of hyperglycemic bovine aortic endothelial cells to ramipril showed a significant increase in sRAGE released into the medium (Forbes et al., 2005). Likewise, in a study involving humans, there was a significant elevation in plasma sRAGE in subjects who had type-1 diabetes and were administered the ACEi perindopril, as compared to those patients who received a placebo or nifedipine, and showed no increase in sRAGE (Jerums et al., 2001).

Telmisartan, an antihypertensive agent that acts by blocking angiotensin II type I receptors (ARBs), has been reported to lower plasma sRAGE levels in patients with essential hypertension (Nakamura et al., 2005). Although ACEi and ARBs both attenuate hypertension by acting on the angiotensin system, their mechanisms of action are different, which may explain their opposite responses concerning sRAGE.

Wendt et al (2005) reported increased development of atherosclerosis in apoE-deficient mice treated with streptozotocin. The streptozotocin-induced diabetic atherosclerosis

was associated with increased aortic expression of VCAM-1, tissue factor and MMP-9 as compared to nondiabetic mice. Diabetic mice treated with sRAGE showed significantly decreased aortic VCAM-1, tissue factor and the activity of MMP-9, although, ICAM-1 was not affected. Treatment of diabetic apoE-deficient mice with sRAGE totally prevented the development of atherosclerosis in a glycemia- and -lipid independent process. Likewise, Bucciarelli et al. (2002) demonstrated that sRAGE significantly attenuates atherosclerotic lesion area in diabetic apoE-deficient mice. They also reported that sRAGE influences atherosclerosis progression in euglycemia. These data suggest that atherosclerosis is decreased in diabetic and nondiabetic animals administered sRAGE and further corroborate the role of this receptor in vascular inflammation.

1.3.2.2 sRAGE and Suppression of Atherosclerosis

AGE accumulates in atherosclerotic plaques and in smooth muscle cells extracted from atherosclerotic plaques (Kume et al., 1995; Horiuchi et al., 1991). AGE interaction with RAGE in vascular SMC leads to the release of transforming growth factor β, resulting in SMC migration and extracellular matrix formation, and hence modulation of atherosclerosis (Higashi et al., 1997). Wendt et al (2000) found that accelerated atherosclerosis in the aortas of streptozotocin-induced diabetic apoE-null mice was associated with increased expression of VCAM-1, tissue factor and MMP-9 as compared to nondiabetic mice. Administration of sRAGE to the diabetic mice resulted in a significant attenuation of aortic VCAM-1, tissue factor and MMP-9. Two separate studies conducted by Park et al. (1998) and Bucciarelli et al. (2002) further demonstrated that sRAGE reduced atherosclerotic lesions in a glycemia- and lipid-independent fashion. These studies imply that RAGE and AGE may be involved in the pathogenesis

of atherosclerosis and that sRAGE may suppress the development of atherosclerotic lesions (Basta, 2008a).

### 1.3.3 AGEs and Cellular Activation

Initially, there was speculation that AGEs attached to senescent proteins, thus enabling a specific signal for the identification, degradation and elimination of these senescent macromolecules by the kidneys (Vlassara et al., 1985; Makita et al., 1991). Contemporary studies, however, have revealed that AGE/RAGE interactions function to break down AGEs and stimulate cellular signal transduction pathways. AGE-induced cellular activation promotes the synthesis and release of cytokines and growth factors that may begin tissue repair and protein remodeling (Vlassara et al., 1995). Moreover, several researchers have shown that there is a positive correlation between AGEs and the advancement of vascular disease, diabetes atherosclerosis and aging (Schmidt et al., 1995b; Vlassara et al., 1994; Vlassara et al., 1995). The damaging effects of AGE formation on the vasculature have dichotomous effects. Firstly, the formation of AGEs on the proteins of the extracellular matrix induces increased vascular thickness and rigidity with associated decreased elasticity and lumen diameter (Bierhaus et al., 1998). Secondly, AGEs produced on the proteins of the vascular matrix cause deficiencies in the vasodilatory activity of the vascular SMC and endothelium by inhibiting nitric oxide (NO) function (Bucala et al., 1991). Endothelial cells control vascular inflammation by mediating the generation of cytokines, chemokines and adhesion molecules. Nitric oxide, a major vasodilator, is produced under the influence of endothelial nitric oxide synthetase (eNOS). Other actions of NO include the inhibition of platelet stimulation and vascular SMC proliferation and migration (Beckman and Creager, 2006). It has been demonstrated that endothelium-dependant vasodilation is reduced in patients with

atherosclerosis (Anderson et al., 1995; Beckman et al., 2003; Liao et al., 1995). The AGE-induced reduction in the effect of NO, combined with the AGE-induced expression of the potent vasoconstrictor endothelin-1, changes the overall vascular tone towards one of vasoconstriction, thereby attenuating the modulation of vascular tone and setting the stage for the development of atherosclerosis (Quehenberger et al., 1995).

Cellular antioxidant defence components (e.g. glutathione, vitamin C) become deficient following the binding of AGEs to their counterreceptors, resulting in production of reactive oxygen species (Esposito et al., 1989; Schmidt et al., 1995b; Vlassara, 1995). Correspondingly, increased oxidative stress results in the activation of the ROS-responsive transcription factor NF-κB which in turn stimulates the expression of NF-κB-controlled genes, such as the procoagulant tissue factor and/ or VCAM-1 that have been linked to the genesis of atherosclerosis (Esposito et al., 1989; Schmidt et al., 1995b; Vlassara, 1995).

1.3.3.1 Activation of Nuclear Factor Kappa B (NF-κB)

NF-κB was identified in 1986, and it exists in biological systems to form a possible pathway for the accelerated transcriptional stimulation of an array of genes encoding for the following: (1) growth factors; (2) adhesion molecules (ELAM-1, VCAM-1, ICAM-1); (3) acute phase proteins (serum amyloid A precursor, angiotensinogen, complement factors B and $C_4$,); (4) transcription factors (NF-κB precursor p105, IkBα); (5) immunoreceptors (tissue factor, immunoglobulin kappa light chain); (6) cytokines (TNF-α, IL-1, MCP-1, chemokines); and (7) nitric oxide synthetase production (Sen and Baltimore, 1986; Schmidt et al., 2000; Schmidt and Stern, 2001; Schmidt et al., 1995a; Miyata and Asahi, 1999; Alderson et al., 2004; Fan et al., 2002; Baeuerle and Henkel, 1994). As reviewed by Prasad (2006a), contemporaneous information has identified

inducible transcription factors of the NF-κB/Rel family of regulator genes in association with cellular inflammation and proliferation in the pathogenesis of atherosclerosis. According to Parhami et al. (1993), NF-κB is activated by minimally modified low-density lipoprotein (MMLDL). Once activated by MMLDL, NF-kB induces the expression of genes that encode for proteins necessary for leukocyte margination and adhesion. This process has been closely linked with the pathogenesis of atherosclerotic plaque formation. Similarly, NF-κB is activated in response to an atherogenic diet in mice (Liao et al., 1993). In addition, in many cells NF-κB stimulation protects from apoptosis through activation of survival genes. Impaired regulation of NF-κB is directly involved in various human diseases such as neurodegenerative disorders (Grilli and Memo, 1999), ataxia-telangiectasia (Lee et al., 1998), arthritis (Foxwell et al., 1998), asthma (Barnes and Adcock, 1998), inflammatory bowel diseases (Neurath et al., 1998) and atherosclerosis (Karin and Ben-Neriah, 2000; Bierhaus et al., 1998).

In the inactive state, NF-κB is a sequestered cytosolic dimer composed of two subunits: p50 and p65 (Rel A). NF-κB is immobilized by the inhibitory complex known as the inhibitor of kB (IkB). NF-κB is stimulated by various extracellular mediators such as: proinflammatory cytokines (TNF-α and IL-1), ROS ($H_2O_2$ and $O_2^-$), phorbol esters, microorganisms, lipopolysaccharides, calcium ionophores and cyclohexamides and Chlamydia pneumonia infections (Schreck et al., 1991; Schreck et al., 1992a; Dechend et al., 1999). Stimulators of NF-κB induce phosphorylation and ubiquination with consequent proteolytic dissociation of IκBα by the 26S proteasome (Whiteside and Israel, 1997; Collins and Cybulsky, 2001). The fundamental function of the 26S proteasome is to hydrolyse the substrate's peptide bonds, thus reducing a folded protein into oligopeptides and ultimately free amino acids which then can be used in new

biosynthetic processes (Hanna and Finley, 1995). The 26S proteasome separates IκBα from the β dimeric complex revealing the positively charged subunits p65 and p50. The currently known subgroups of the NF-κB family in mammals are p50, p65/Rel A, p52, c-Rel, and Rel B. In mammals, various types of the inhibitory complex IκBs are present such as IκBα, β, γ (p105), δ (p100), ε, and BCL-3 (Bowie and O'Neill, 2000a). However, the most in-depth research has been centered on the p50, p65/Rel A subunits of the NF-κB family and its correlated inhibitory complex IκBα. Directly following activation, NF-κB (p50/65) is released from its inhibitory complex, and then translocated to the nucleus where it binds with regulatory κB constituents in promoters and enhancers of DNA, thus determining gene expression (Henke et al., 2007). The activation of NF-κB is analogous to the turning on of a switch for the transcription of various gene families encoding cytokines, cytokine receptors, cell adhesion molecules, acute-phase proteins and growth factors (Baeuerle and Henkel, 1994; Thanos and Maniatis, 1995; Hachiro et al., 1996)

1.3.3.2 The Primary Elements in NF-κB Activation

NF-κB inducers act on the inhibitory complexes IκBα and IκBβ precisely at their two serine residues: $Ser^{32}$ and $Ser^{36}$, and $Ser^{19}$ and $Ser^{23}$, respectively. These particular serine residues are the sites for phosphorylation and ubiquination, and successive degradation by the proteasome 26S (Whiteside and Israel, 1997). The ubiquination and phosphorylation of IκBα are closely linked and are of significant importance. These biochemical processes function to create chemical targets or markers for degradation of IκB by the 26S proteasome.

*1.3.3.2.1 Ubiquination of IκB*

The ubiquination proteolysis system was initially thought to function as a mechanism to degrade senescent, misfolded or misassembled proteins. The system derived its name from the small protein called ubiquitin which is found in large stores in all eukaryotes. The ubiquination proteolysis system has been linked to the control of various functional regulatory proteins such as the following: (1) oncoproteins; (2) transcription factors; (3) cell growth modulators; (4) signal transducers; and (5) cell cycle proteins (Karin and Ben-Neriah, 2000; Baumeister et al., 1998; Varshavsky, 1997). The ubiquitin pathway is composed of many constituents functioning in a concerted manner to produce a high-energy thioester linkage between ubiquitin and activating enzymes (E1-E3 ligases) (Haas and Siepmann, 1997), which attach ubiquitin molecules to the ε amino group of lysine residues of IκBα (Karin and Ben-Neriah, 2000; Baumeister et al., 1998; Varshavsky, 1997). The initial action for degradation of the inhibitory complex (IκBα) is the covalent bonding of multiple ubiquitin molecules to the substrate. This occurs in three consecutive steps: (1) covalent bonding of one or more ubiquitin polypeptides to the protein substrate; (2) ubiquitin-ubiquitin conjugation to generate ubiquitin polymers; and (3) degradation of the ubiquitin-labelled protein by the 26S proteasome complex (Karin and Ben-Neriah, 2000). Thus, the formation of an ubiquitin chain functions as a recognition target for degradation by the 26S proteasome leading to unmasking and activation of NF-κB.

*1.3.3.2.2 Phosphorylation of IκB*

Signals from the cytokines (IL-1 and TNF) have been shown to activate NF-κB through interactions with their complementary cell surface receptors (Sims et al., 1993; Auron, 1998; Bowie and O'Neill, 2000a). These signalling pathways are concentrated and

coordinated at a multi-subunit IkB complex that consists of two highly homologous catalytic subunits: IκB-kinases (IKKs), termed IKKα and IKKβ. The function of IKK enzymes is to mediate NF-κB activation by phosphorylation of IκBα at the serine residues $Ser^{32}$ and $Ser^{36}$ (DiDonato et al., 1997). The IKKs are a component of a signalsome that is regulated by IKKγ, also called NF-κB essential modulator (NEMO), and IKK-associated protein-1 (IKKAP1) (Kempe et al., 2005; Karin and Ben-Neriah, 2000). A recently identified protein called ELKS, whose name was derived from the abundance of its amino acid groups, glutamic acid (E), leucine (L), lysine (K) and serine (S), was formerly known as a pervasive protein of unknown function. However, Sigala et al. (2004) have suggested that ELKS mediates an important step in IKK activation. Rudolph et al. (2000) have demonstrated that mice lacking IKKβ or NEMO experience impaired cytokine-influenced NF-κB stimulation. The mechanism of action for the activation of IKKs by the proinflammatory cytokine TNF-α is as follows: first, TNF-α binding to its associated receptor TNF-α receptor1 (TNFR1) induces receptor clustering with subsequent TNFR1 interaction with cytosolic TNFR1-associated death domain protein (TRADD) (Bowie and O'Neill, 2000a; Baeuerle, 1997; Karin and Ben-Neriah, 2000). Next, TRADD has a role in the recruitment of TNFR-associated factor 2 (TRAF2) receptor interacting protein (RIP), and a serine/threonine kinase, which are necessary for the activation of NF-κB. Finally, TRAF2 interacts with and stimulates NF-κB inducing kinase (NIK) that in turn activates IKKs (Bowie and O'Neill, 2000a; Baeuerle, 1997; Karin and Ben-Neriah, 2000). Conversely, the proinflammatory cytokine IL-1 activates NF-κB by stimulation of IKKs through a different pathway. IL-1 reacts with its receptor IL-1R which induces receptor clustering and recruitment of marker proteins from their cytoplasmic domains. Next, IL-1R recruits the IL-1R

accessory protein (IL-1RACP) that goes on to attract IL-1R-associated kinase (IRAK), through the adaptor molecule MyD88 (Wesche et al., 1997; Cao et al., 1996a; Muzio et al., 1997). IRAK then activates a protein analogous to TRAF2 called TRAF6 (Cao et al., 1996b) which may couple with the mitogen-activated protein kinase kinase kinase (MAPKKK) leading to the stimulation of TGF-β-activating kinase-1. Finally, TGF-β-activating kinase-1 directs the activation of NIK which is responsible for the activation of IKK (Ninomya-Tsuji et al., 1999). The exact mechanism by which TRAF activates IKK is not clear. However, it is thought that clustering of the N-terminal domain produces a recognition region platform for a downstream signalling protein (Karin and Ben-Neriah, 2000). Although the proinflammatory cytokines TNF-α and IL-1 both stimulate NF-κB through IKK by different pathways, the common component appears to be the utilization of TRAF proteins (Karin and Ben-Neriah, 2000). Nevertheless, the function of IKK enzymes is to mediate NF-κB activation by phosphorylation of IκBα at the serine residues $Ser^{32}$ and $Ser^{36}$ leading to the eventual degradation of IκB and translocation of NF-κB from the cytosol to the nucleus for gene transcription (DiDonato et al., 1997; Karin and Ben-Neriah, 2000; Prasad, 2006a; Bowie and O'Neil, 2000a).

The Oxidative Stress Theory of NF-κB Activation

The control of gene expression by oxidants, antioxidants and the redox state has interesting therapeutic implications for the control of disease states associated with NF-κB activation, particularly atherosclerosis (Bowie and O'Neill, 2000a). The model of the oxidative stress-induced activation of NF-kB suggests that diverse agents can activate NF-κB. The activation of NF-κB is accomplished by increasing oxidative stress through elevation in intracellular reactive oxygen species such as $H_2O_2$, superoxide and hydroxyl radicals (Meyer et al., 1993). The evidence to support the oxidative stress-

induced activation of NF-κB is as follows: (1) the addition of hydrogen peroxide alone to culture medium can activate NF-κB in some cells lines; (2) antioxidants such as pyrrolidine dithiocarbamate (PTDC), N-acetyl cysteine (NAC), α-lipoate, butylated hydroxyanisole (BHT) and desferrioxamine (DFO) have all been demonstrated to inhibit NF-κB activation; (3) in certain cell lines ROS have been shown to increase in responses to substances that induce NF-κB; and (4) enzymes which adjust the redox status of the cell such as catalase, glutathione peroxidases and superoxide dismutases may either increase or decrease the activation of NF-κB (Schreck et al., 1992b; Schreck et al., 1991; Meyer et al., 1993; Schmidt et al., 1995; Los et al., 1995; Manna et al., 1998; Brigelius-Flohe et al., 1997; Bonizzi et al., 1999). The oxidative stress model postulates an explanation of how differing biochemicals could activate NF-κB through increasing ROS within the cell. However, no common redox-sensitive step has been demonstrated to converge in the signalling of IkB kinases which appear to be central to NF-κB activation (Bowie and O'Neill, 2000b).

## 1.4 Theories of Atherosclerosis

Although there are several theories of the genesis of atherosclerosis they all have a central concept centered on the following factors: (1) lipid involvement in the formation of a lesion; (2) monocyte migration and proliferation; and (3) smooth muscle proliferation.

### 1.4.1 Response to Injury Hypothesis

The most commonly accepted theory of atherosclerosis is the response to injury hypothesis. It was first proposed by Virchow in 1877 and described by Rather in 1956. The response to injury theory was later modified and expanded by Ross and Glomset (1976), and subsequently updated by Ross in 1986. This theory of atherosclerosis is

based upon endothelial damage caused by chemical, mechanical, immunological or toxicological insult. Endothelial damage leads to endothelial dysfunction, which initiates a lesion and causes exposure of the subendothelial tissue. These events may be responsible for the insudation of plasma lipoproteins into the arterial wall. Furthermore, the progression of the lesion involves monocyte and platelet chemotaxis and adherence to the endothelium (Faruqui et al., 1994). The formation of endothelial gaps (20-60 nm) may be due to structural changes in the microvasculature where there is endothelial cell contraction, or intercellular endothelial cell retraction and cytoskeletal reorganization under the influence of interleukins, TNF-α and interferon. Next, monocytes, and T lymphocytes undergo diapedesis and transmigrate through the endothelium into the extravascular space where monocytes are subsequently converted into mature macrophages. Tissue macrophages phagocytize lipids, and become immobile foam cells which form the early atherosclerotic fatty streak lesions (Ross, 1993).

The adhesion of white blood cells to the endothelial surface is accomplished through endothelial-dependent counterreceptor mechanisms. The increase in recruitment and adhesion of monocytes and T lymphocytes is due to endothelial-dependent counterreceptor mechanisms which form specific sets of adhesion molecules. To provide selective adhesion between monocytes and the endothelium, monocytes express the receptor very late antigen-4 (VLA-4). Next, VLA-4 unites with its vascular endothelial counterreceptor, VCAM-1 (Gimbrone, 1995). There are various other endothelium-dependent counterreceptor systems contributing to the increase in adherence of monocytes to the endothelium, such as the selectin and integrin varieties: (1) ICAM-1 which interacts with CD11/CD18-integrin complex; (2) endothelial-derived

MCP-1; (3) IL-1; (4) TNF-α; and (5) hypercholesterolemic serum lipoproteins (Gimbrone, 1995; Denholm and Lewis, 1987; Chin et al., 1992).

Prasad (2003) described several growth modulators produced by macrophages, smooth muscle cells, endothelial cells, and T-lymphocytes which have paracrine and/or autocrine effects. Paracrine effects are expressed when growth modulators act upon other cell types in the vicinity. However, an autocrine effect is manifest when growth modulators act upon the cells that have produced them (Denholm and Lewis, 1987). Macrophages produce the following growth modulators which have paracrine effects: (1) platelet-derived growth factor (PDGF); (2) heparin-binding epidermal growth factor (HB-EGF); (3) cytokines (IL-1, TNF-α); (4) oxidized low density lipoproteins (OxLDL); and (5) basic fibroblast growth factor and transforming growth factors α and β which all modulate the action of adjacent cells (Hansen et al., 1994; Gimbrone, 1995). Furthermore, TGF-β which is also produced by T lymphocytes has two main functions: (1) inhibition of SMC replication; and (2) initiation of the production of connective tissue and matrix such as elastic fiber proteins, collagens and proteoglycans. In addition, smooth muscle cells form OxLDL, macrophage colony stimulating factor (M-CSF) and granulocyte-monocyte colony stimulating factor (GM-CSF), all of which induce monocyte differentiation into mature macrophages (Denholm and Lewis, 1987). Colony stimulating factor plays an important part in macrophage immobility and replication. According to Prasad (2000a), smooth muscle and endothelial cells also control growth by the production of PDGF, basic fibroblast growth factor (bFGF), TGF, insulin-like growth factor I (IGF-I), and cytokines (IL-1, TNF-α). Similarly, cytokines exert an autocrine effect on smooth muscle by stimulating SMC proliferation, and a paracrine effect by eliciting endothelial activation (Prasad, 2000a).

In summary, the response to injury hypothesis is based upon insult by hyperlipidemia or other risk factors that induce endothelial injury and dysfunction causing platelet and monocyte adhesion, and liberation of cytokines, chemo-attractants, and growth regulators. This sequence of events triggers smooth muscle cell proliferation, chemotaxis and emigration of more monocytes with eventual macrophage lipid retention creating a vicious cycle that leads to the genesis of the atherosclerotic fatty streak lesion (McNair et al., 2006; Hanahan, 1986).

### 1.4.2 Oxidative Hypothesis

In the oxidative hypothesis, the production of the atherosclerotic fatty streak lesion is due to the oxidation of low-density lipoprotein cholesterol (LDL-C), which then accumulates in immobile tissue macrophages termed foam cells (Henriksen et al., 1981). Plasma lipoprotein and LDL-C set the stage for atherogenesis through oxidation by smooth muscle cells, macrophages and endothelium (Keany and Vita, 1995). The oxidation of LDL-C to minimally modified LDL-C (MMLDL) stimulates endothelial and smooth muscle cells to produce monocyte chemotactic protein 1 (MCP-1) which in turn increases monocyte migration to the subendothelial area (Chisolm, 1991). MMLDL is likewise oxidized to OxLDL which can directly cause endothelial damage, trigger additional monocyte migration, and stimulate the release of MCP-1 and M-CSF from endothelial cells. OxLDLs also serve as ligands for the scavenger receptor of macrophages (Steinberg, 1991), as outlined by Prasad (2000a). Thus, macrophage engulfment of OxLDL converts them into OFR-releasing, immobile foam cells, stationary at the focus of the lesion (Rapava et al., 2006). Finally, the endothelial adhesion of monocytes depends upon cytokines and chemoattractants such as IL-1β and

M-CSF, which affect surface expression of endothelial–leukocyte adhesion molecules (ELAMs) (Schwartz et al., 1993; McNair et al., 2006).

### 1.4.3 Advanced Glycation End Product Hypothesis

The most recent theory of atherosclerosis involves AGEs in the genesis and progression of the atherosclerotic lesion. The attachment of AGEs to their receptors (RAGEs) changes a short pulse of cellular activation to continued cellular dysfunction and tissue destruction which is associated with the pathogenesis of atherosclerosis (Bierhaus et al., 2005). The predominance of AGE receptors at sites of vascular injury may play a critical role in the pathogenesis of vascular lesions (Gugliucci, 2000; Schmidt et al., 1999). It is known that oxidation of the lipid segment of LDL plays an integral role in the genesis of atherosclerosis. Elevated glucose levels as seen in diabetics may explain the biochemical mechanism that produces lipid oxidation. Lipid-advanced glycosylation (lipoxidation) products and oxidized LDLs are elevated during chronic hyperglycemia of diabetes, which supports the hypothesis that AGE oxidation functions in stimulating lipid oxidation (Bucala et al., 1993). The glycosylation of lipids subsequently promotes free radical formation that activates the ROS-sensitive NF-κB. NF-κB stimulation also causes a positive feedback loop in which augmented RAGE expression increases the endothelial binding of more AGEs to RAGEs (Schmidt et al., 1999). NF-κB activation also results in the production of genes that are involved in the production of proinflammatory cytokines (IL-1 and TNF-α), endothelial adhesion molecules, prothrombotic molecules (tissue factor), vasoconstrictive molecules (endothelin-1) and the molecule RAGE itself (Bierhaus et al., 2005). Thus, the AGE-induced oxidation of LDL resulting in modifications in intimal collagen is a crucial step toward the initiation of the proinflammatory chain of events that results in the pathogenesis of the

atherosclerotic lesion (Basta et al., 2004). According to the oxidation theory of atherogenesis, subintimal LDLs become lightly oxidized to become minimally-modified LDL, which is further oxidized to become oxidized LDL (OxLDL) (Prasad, 2000a). Bucala et al. (1993) and Vlassara (1996) demonstrated that elevated levels of glycosylated LDL in diabetics result in OxLDL formation. Moreover, scavenger receptors for OxLDL are present on macrophages. Macrophages engulf OxLDL and eventually form immobile foam cells in the developing lesion (Steinberg, 1991). Interestingly, Vlassara et al. (1986) identified unique receptors for AGEs on monocytes and macrophages. It is possible that atheromatous lesions that are formed from the macrophages (foam cells) that are immobilized at the focus of the lesion may contain AGE-LDLs (Miyata and Asahi, 1999). Cerami et al. (1986) demonstrated that LDL is sequestered explicitly by AGEs produced by the nonenzymatic glycosylation of collagen, which implies that increased LDL trapping by AGEs may augment the progression of atherosclerosis in diabetic patients. The nonenzymatic glycosylation of collagen results in the following: (1) protein accumulation and thickening of the basement membrane; (2) capture of IgG with subsequent complement activation; and (3) attenuation of the vasodilatory effect of nitric oxide (Bucala et al., 1995; Cerami et al., 1985; Cerami et al., 1986). Moreover, AGEs have the ability to covalently cross-link and form irreversible intermolecular bonds to proteins on collagen, thereby contributing to the pathogenesis of focal sclerosis and mesangial expansion seen in diabetic glomerulopathies and the presence of vascular complications (Hunt et al., 1990). Consequently, the deleterious results from the biological activities of AGEs are cross-linking of proteins, mutagenesis of nucleotides, and the initiation of lipid peroxidation with the genesis of ROS (Hunt et al., 1990). AGE concentrations are markedly elevated

in subjects with hyperglycemia because their production increases at a greater rate than the increase in the blood glucose level (Giardino et al., 1994). To reiterate, AGEs can be produced in two major ways: through the primary nonenzymatic action of sugars on proteins; and through oxidative reactions of early glycation products. According to Baynes and Thorpe (2000), the theory of "carbonyl stress" may best describe the glycoxidation (nonoxidative) and lipoxidation (oxidative) of tissue proteins associated with diabetes. They implied that in diabetes, sugars and lipids increased the conversion of proteins to AGEs by overwhelming the metabolic pathways used to detoxify reactive carbonyl molecules. This increase in glycoxidation and lipoxidation products leads to an increase in the plasma levels of reactive carbonyl compounds (Thomas et al., 2005).

It has also been demonstrated by Cerami et al. (1986) that AGEs are chemotactic for human mononuclear leukocytes and they induce selective transendothelial migration of monocytes through the endothelial cell surface. Bucala et al. (1993) postulated that long-term application of *in vitro* synthesized AGE-albumin resulted in vascular defects, vascular permeability, inadequate vasodilation, and activation of NF-κB, with consequent adhesion molecule expression (VCAM-1) for recruited monocytes (Schmidt et al., 1995b). Finally, the contributions of AGEs to the development and progression of atherogenesis are multifactorial, including the following: (1) increased vascular matrix formation with decreased lumen diameter (Rumble et al., 1997); (2) increased basement membrane deposition (Brownlee et al., 1988); (3) increased membrane permeability (Esposito et al., 1989); (4) stimulation of cytokines and growth factors (Kirstein et al., 1992; Yang et al., 1994); (5) increased smooth muscle cell proliferation (Hogan et al., 1992); (6) augmented procoagulant action (tissue factor expression) (Esposito et al., 1989; Bierhaus et al., 1997a); (7) activation of RAGE (Bierhaus et al., 1997a); (8)

stimulation of vascular endothelial growth factor production (Yamagishi et al., 1997); (9) inhibition of anticoagulant functions (thrombomodulin activity) (Esposito et al., 1989); (10) stimulation of MCP-1 in smooth muscle cells (Bierhaus et al., 1998); (11) suppression of nitric oxide activity, thereby decreasing the smooth muscle vasodilation (Bucala et al., 1991); (12) augmented vasoconstriction by the stimulation of endothelin-1 (Quehenberger et al., 1995); and (13) attenuation of antioxidant defence systems (e.g. vitamin C and reduced glutathione) (Bierhaus et al., 1997b; Nishikawa et al., 2000; Aronson, 2008). AGEs stimulate a multitude of cell-mediated responses that may contribute to the pathogenesis of atherosclerosis and could be the link to unify and substantiate the differing theories of diabetic and nondiabetic vascular and coronary artery disease.

**1.5 C-Reactive Protein (CRP) and Atherosclerosis**

A review by Prasad (2003) characterized C-reactive protein as an acute phase reactant protein that serves as a novel biomarker for inflammation and atherosclerosis. Generally, it is a risk factor for cardiovascular disease and may specifically predict the development of MI and stroke in healthy individuals (Pearson et al., 2003; Ridker et al., 2008; Ridker, 2008). It also functions as an active intermediary of atherosclerosis by stimulating arterial endothelial activation and macrophage recruitment (Zhong et al., 2006). Furthermore, CRP downregulates endothelial NO synthetase protein expression and diminishes endothelial NO synthetase mRNA, producing decreased release of basal and stimulated NO (Verma et al., 2002a). According to Gauldie et al. (1987), the stimulation of monocytes and macrophages during inflammation induces them to release cytokines such as IL-6, IL-1β, TNF-α, interferon λ and transforming growth factor β. Cytokines in turn stimulate hepatocytes to release the major positive acute phase

proteins CRP and serum amyloid A. Those proteins whose plasma levels increase by at least 25% during inflammatory conditions are termed positive acute phase proteins. Those proteins whose plasma concentrations decrease by at least 25% are termed negative acute phase proteins (Morley and Kushner, 1982). The major negative acute phase proteins are albumin, transferrin and transthyretin (Kushner and Feldmann, 1978). CRP elicits numerous reactions such as complement activation, accelerated neutrophil phagocytosis and release of ROS, stimulation of adhesion molecules and plasminogen activator-1, plaque destabilization, uptake of LDL by macrophages to form foam cells, production of tissue factor, and increases in platelet aggregation (Pearson et al., 2003; Prasad, 2006b). A postulated primary proinflammatory function of CRP is related to its affinity for phosphocholine (PC), a host microbe ligand that is expressed on the phospholipid components of damaged cells and pathogens. Through an innate immune response, the binding of CRP to PC allows for the detection of foreign pathogens by activation of the classical complement pathway, thereby inducing the elimination of these target cells (Volanakis, 1997; Hoffmann et al., 1999). CRP has anti-inflammatory roles as well. For instance, one study demonstrated that increased CRP levels caused a detachment of L-selectin, thereby decreasing endothelial neutrophil adhesion (Zouki et al., 1997). Prasad (2003) indicated that CRP can enhance the expression of adhesion molecules by increasing oxidative stress and decreasing antioxidant production. Gershov et al. (2000), however, have shown that CRP-stimulated macrophage engulfment of apoptotic cells, and the attachment of CRP to lymphocytes subjected to apoptosis inhibited complement activation and lysis.

CRP can cause atherogenesis by the production of oxygen free radicals and the expression of adhesion molecules (Prasad, 2003; Prasad, 2004). The oxidative theory of

atherosclerosis is based upon the pathophysiological generation of ROS. CRP directly stimulates monocytes, PMNLs and neutrophils to produce ROS, and indirectly induces ROS production by complement activation, stimulation of platelet activating factor and expression of cytokines (Prasad, 2006b). Moreover, CRP also induces the production of MCP-1, IL-6, IL-8, ICAM-1, and VCAM-1 by arterial endothelial cells (Ridker et al., 1998a; Verma et al., 2002b; Pasceri et al., 2001; Devaraj et al., 2004). Recently, it has been reported that CRP levels known to predict the risk of future cardiac events, upregulates RAGE expression at the protein and mRNA level in human endothelial cells. Furthermore, CRP stimulates RAGE ligand binding which implies that elevated CRP levels can induce the cellular activation associated with atherosclerotic lesion formation. The suppression of RAGE expression re-establishes the CRP-induced MCP-1 activation, indicating that CRP raises MCP-1 concentrations through the RAGE pathway (Zhong et al., 2006).

There appears to be a body of evidence indicating that levels of CRP are correlated with serum lipids (Prasad, 2006). Elevated levels of CRP have been shown in LDL hypercholesterolemia resulting from cholesterol-supplemented diets. Conversely, low CRP levels are observed in dietary cholesterol-deficient diets (Verhamme et al., 2002). In addition, elevated plasma lipoprotein (a) [Lp (a)] is correlated with increased concentrations of CRP. Several studies have demonstrated that hydroxymethylglutaryl coenzyme A reductase inhibitors (statins) can be used to reduce plasma CRP levels in patients with hyperlipidemia (Moon and Kashyap, 2002; Verhamme et al., 2002; Ridker et al., 1998a; Prasad, 2006b). The incorporation of niacin and lovastatin administered to patients with hyperlipidemia lowers serum LDL and triglycerides (TG), and decreases Lp (a) and CRP by 25% and 24%, respectively (Moon and Kashyap, 2002). Recently, it

has been demonstrated by Zhong et al (2006) that CRP levels known to predict the risk of the development of potential vascular events upregulates RAGE expression in human endothelial cells at both the protein and mRNA stages. In addition, silencing of the RAGE gene prevents CRP-induced MCP-1 activation and thus attenuates the deleterious effects of the AGE-RAGE interaction.

**1.6 Oxygen Radicals and Atherosclerosis**

Prasad (2000b) described the function of ROS in the pathogenesis of atherosclerosis. Within an atom there is a nucleus surrounded by orbitals that contain spinning electron pairs. A free radical is an extremely reactive atom or molecule with a short half-life and has one or more unpaired electrons in its outer orbital. An oxygen free radical (OFR), on the other hand, a compound that is produced from molecular oxygen, has fewer than four electrons in its outer orbital. Reactive oxygen species (ROS) such as hydrogen peroxide and hyperchlorous acid are derived from molecular oxygen, and they contain an even quantity of electrons in their outer shells and are not free radicals (Lindqvist and Nordstrom, 2001). ROS have been shown to contribute to the development of various human diseases such as hypercholesterolemic atherosclerosis, ischemia-reperfusion injury, peripheral vascular disease, heart failure, diabetes mellitus, Parkinson's disease, Alzheimer disease, multiple sclerosis, amyotrophic lateral sclerosis (ALS), hemorrhagic and endotoxic shock, burns, cataracts, rheumatoid arthritis, asthma and cancer (Prasad et al., 1989; Prasad et al., 1990; Prasad et al., 1991; Prasad et al., 1992a; Prasasd et al., 1992b; Prasad and Kalra, 1993; Prasad et al., 1993; Prasad et al., 1994; Kapoor and Prasad, 1996; Prasad and Bharadwaj, 1996; Prasad et al., 1996; Prasad, 1997a; Prasad, 1997b; Prasad et al., 1997; Prasad, 1998; Prasad et al., 1998; Pattanaik and Prasad, 1998; Prasad, 1999; Prasad, 2005; Babior, 1984; Baynes, 1991; Lindquist and

Nordstrom, 2001; Lehr et al., 2001). The oxidative hypothesis of atherosclerosis is based upon endothelial injury from oxidation of LDL (Henriksen et al., 1981). The oxidation of LDL could be due to the production of ROS (Keany and Vita, 1995). Interestingly, endothelial injury also causes complement activation ($C_3$ and $C_5$) (Webster et al., 1980). Activated complement, platelet-activating factor (PAF), leukotriene $B_4$ ($LTB_4$) and TNF stimulate PMNLs to release oxygen free radicals (Ford-Hutchinson et al., 1980; Braquet et al., 1989; Zoratti et al., 1991; Paubert-Braquet et al., 1988).

1.6.1 Sources of Oxygen Radicals

ORs are produced under physiological and pathophysiological conditions throughout the body.

*Mitochondria*

The primary location for $O_2^-$ formation is the mitochondrial ubiquinone-cytochrome b region of the electron transport chain. In this univalent process, oxygen is reduced to $O_2^-$ and receives one electron at a time (Southorn and Powis, 1988). Under physiological conditions only 5% of the inhaled oxygen is metabolized via a univalent process into highly reactive ORs (Fridovich, 1978). A tetravalent pathway producing no reactive oxygen species metabolizes the remaining 95%. The production of $H_2O_2$ is formed from the dismutation of $O_2^-$. "In the Fenton reaction, $H_2O_2$ in the presence of ferrous or cupric ions, is converted to $^{\cdot}OH$" (Prasad 2000a).

*Polymorphonuclear Leukocytes (PMNLs)*

Polymorphonuclear leukocytes contain the NADPH oxidase system which is a membrane-bound flavoprotein complex that functions as an electron transport chain. The NADPH oxidase system is quiescent in uninduced PMNLs. However, upon PMNL receptor stimulation by specific ligands (activated complement, N-formyl- Met-Leu-Phe,

immune complexes, and $LTB_4$), this system catalyzes the reduction of molecular oxygen to superoxide anion. In addition, the dismutation of $O_2^-$ to $H_2O_2$ provides a key component for the synthesis of hypophalous acid (a potent oxidizing agent). According to Kapoor and Prasad (1996), azurophilic granules located within the PMNL contain myeloperoxidase (MPO) which is released into the phagocytic vacuole upon excitation of PMNLs. HOCl is the most potent and abundant hypophalous acid and it is produced primarily by PMNLs.

*Enzymes*

Prasad (2000b) delineated the enzymatic sources of ORs. Under physiological conditions, 80% of the metabolized molecular oxygen is transformed into $H_2O_2$ through a divalent reaction. However, 20% is transformed into superoxide anions through univalent reduction. Xanthine oxidase and aldehyde oxidase are important enzymes that produce ORs from the metabolism of $O_2$ during ischemic tissue injuries (Engerson et al., 1987; McCord and Roy, 1982; McCord, 1985).

*Xanthine Oxidase and Ischemia*

As explained by Prasad (2000b), the biochemical reactions that occur during ischemia induce the formation of ORs. In non-ischemic tissue, xanthine dehydrogenase is an enzyme that is responsible for catalyzing the reduction of $NAD^+$ to NADH without the production of any ORs. However, during myocardial ischemia there are increases of xanthine and xanthine oxidase, which could lead to the formation of oxygen radicals (Jennings and Reimer, 1982; Chambers et al., 1985). During ischemia-reperfusion, xanthine dehydrogenase is transformed into xanthine oxidase by proteases and sulfhydryl group oxidation. As described by Prasad (2000b), the ischemic-induced catabolism of adenosine triphosphate (ATP) to adenosine diphosphate (ADP), adenosine

monophosphate (AMP) and inosine results in hypoxanthine and xanthine formation (McCord, 1985; McCord et al. 1985). The combination of xanthine and xanthine oxidase in the presence of molecular oxygen produces the superoxide anion and hydrogen peroxide (Engerson et al., 1987; McCord and Roy, 1982; Fantone and Ward, 1982).

Vanden (1980) indicated that the decrease in pH during ischemia stimulates phospholipase $A_2$ which would increase the production of arachidonic acid from membrane phospholipids and synthesis of prostaglandins and leukotrienes. Moreover, oxygen radicals are formed during the synthesis of prostaglandins and leukotrienes (Panganamala et al., 1976: Murota et al., 1990). It has been shown by Rao et al. (1983) that ischemia without reperfusion can produce moderate quantities of ORs from oxygen located within the lipid bilayer of cell membranes. However, direct and indirect evidence supports the contention that the burst of OR generation is not observed until reperfusion. The direct evidence has been demonstrated by electron spectroscopy of serum levels of ORs obtained prior to and after reperfusion of myocardial tissue, which showed limited generation of ORs during ischemia, an increase in OR production in the beginning period of reperfusion, followed by a decrease in production for many hours afterwards (Zweier et al., 1987; Bolli et al., 1989). Similarly, indirect support for the reperfusion oxidative burst may be evidenced by numerous studies which show that inhibitors or scavengers of ORs and anti-neutrophil agents administered during reperfusion attenuated serum levels of ORs (Simpson et al., 1988; Barroso-Aranda et al., 1988; Barroso-Aranda and Schmidt-Schonobein, 1989; Kapoor and Prasad, 1996). Furthermore, endogenous antioxidants such as SOD, catalase, glutathione peroxidase and glutathione are depleted, thus decreasing the inherent myocardial antioxidant

defense mechanisms. Consequently, ischemia promotes the biochemical circumstances for tissue damage due to OR production (Kapoor and Prasad, 1994).

*Arachidonic Acid*

The production of arachidonic acid by phospholipases from cell membrane phospholipids and its subsequent metabolism diverges into two distinct pathways. The lipoxygenase pathway producing leukotrienes and the cyclooxygenase pathway forming prostaglandins both generate ORs. During the transformation from prostaglandin $G_2$ ($PGG_2$) to prostaglandin $H_2$ ($PGH_2$), and from $PGH_2$ to thromboxane $A_2$ ($TxA_2$), $OH^-$ and $O_2^-$ are produced. Likewise, $OH^-$ is produced from the lipoxygenase pathway of arachidonic acid metabolism (Steward et al., 1990; Kontos et al., 1980; Salvador et al., 1977).

1.6.2 Other Sources of Oxygen Radicals

According to Prasad (2000b), there are various other sources of OR production such as the auto-oxidation of small molecules (catecholamines, flavins, hydroquinones and thiols) and sites such as the endoplasmic reticulum, nuclear membrane, macrophages and peroxisomes (Freeman and Crapo, 1982; Muller and Sies, 1987).

**1.7 Percutaneous Coronary Intervention (PCI)**

Percutaneous coronary intervention is a procedure in which a high-grade coronary stenosis (plaque) is treated with a percutaneous catheter system [balloons (PTCA), lasers, rotational atherectomy (RA), directional coronary atherectomy (DCA) devices, brachytherapy, bare metal stents (BMS), drug eluting stents (DES)] to diminish, and/or prevent the ischemic effects of coronary stenosis (Serruys et al., 1994; Sigwart et al., 1987). For the purposes of this study, PCI only refers to the dilation of a coronary stenosis using a balloon-tipped catheter system with the implantation of a bare metal

stent in the area of the lesion to diminish and/or prevent the ischemic effects of coronary stenosis. Inflation of the balloon-tipped catheter to a desired atmospheric pressure results in intra-luminal radial forces. These forces produce plaque compression. However, the primary change in lumen geometry is caused by endothelial denudation, fracture and separation of plaque from the underlying medial and adventitial layers (Fischman et al., 1994). Although PCI provides an increased lumen for blood flow with initial reductions in arterial stenoses, the endothelial injury that results may induce two important adverse outcomes of PCI; acute vessel closure and restenosis. Acute vessel closure is usually a result of vessel dissection and/or thrombus formation in about three to five percent of cases, and it typically occurs within the first 24 hours of the procedure (O'Meara and Dehmer, 1997). The clinical events resulting from acute vessel closure are myocardial infarction and death (O'Meara and Dehmer, 1997). However, the hyperplastic response also known as restenosis is the most common adverse outcome of PCI, and will occur over a period of months post-PCI. The clinical restenosis rate for PCI is 25-45% (Bengtson et al., 1990; Ruygrok et al., 2001; Cannan et al. 1999; McNair, 2006)

### 1.7.1 Evaluation of Lesion Severity

Coronary angiography is a dependable method for assessing lesion severity. However, it may be inconclusive in borderline (40 to 60 %) lesions and may require the use of any of the following: (1) IVUS; (2) quantitative coronary angiography (QCA) (computer based system); (3) coronary flow reserve; and (4) fractional flow reserve (Kini, 2006).

*Intravascular Ultrasound*

Intravascular ultrasound (IVUS) is the gold standard for identifying vessel wall anatomy and plaque morphology (Di Mario et al., 1998). Newer IVUS catheters have outer

diameters of 2-3 french and may be introduced via a 6 french guiding catheter. Essentially, the catheter is advanced distal to the lesion and pulled back gradually or by a motorized device allowing a three-dimensional illustration of the vessel wall. In addition, IVUS is of great value in the assessment of optimal or suboptimal stent positioning and stent expansion (Di Mario et al., 1998). However, the drawbacks to IVUS utilization are the cost of the catheter and associated equipment, and its inability to offer information concerning the coronary microcirculation (Di Mario et al., 1998).

*Quantitative Coronary Angiography (QCA)*

QCA is a computer-assisted method which affords an accurate objective evaluation of absolute and relative coronary artery dimensions during angiography. Additionally, QCA is a useful tool for the assessment of coronary lesion morphology in the estimation of short-and long-term angiographic outcomes following PCI. The benefit of QCA over other methods of lesion evaluation is the capacity to gauge lesion severity without crossing the lesion with a guide wire or other devices. However, one of the drawbacks of QCA is that it also gives no information concerning the coronary microcirculation (Reiber et al., 1985).

*Coronary Flow Reserve (CFR)*

Coronary flow reserve is the capacity to increase coronary blood flow (CBF) from resting CBF to increased CBF by decreasing the vasomotor tone of the resistance vessels (coronary arterioles). This phenomenon occurs in response to increased myocardial demand (hyperemia) (Gould and Lipscomb, 1974). Upon increased myocardial demand, healthy individuals may increase CBF 3 to 5 fold. Coronary lesions that are physiologically significant induce the resistance vessels to dilate, thereby compensating for a decreased CBF. However, with a severe lesion the resistance vessels are fully

dilated and no further dilation is possible. Consequently, under these circumstances the introduction of a physiologic or pharmacologic stimulus that increases myocardial demand will not result in an increased CFR. Essentially, CFR begins to decrease with a stenosis of 50% or greater. Thus, it is accepted that lesions of 70% or greater correspond to significant obstructive coronary artery disease (Kini, 2006; Kern and Meir, 2001). By obtaining the maximum flow velocity (using a 0.014-inch intracoronary Doppler guide wire), and dividing it by the basal flow velocity during hyperaemic vasodilation, the CFR can be calculated. Pharmacological agents such as adenosine and dipyridamole which act mainly on vessels less than 200 μm in diameter are used to evaluate the changes in coronary microcirculation as reflected by changes in coronary flow (Lim et al., 2000; Kini, 2006). Research into the physiological assessment of intermediate lesions has shown that cardiac interventionalists may safely delay performing a PCI on patients who have normal physiologic parameters (normal values of CFR are greater than 2.5) (Kern et al., 1995; Serruys et al., 1997; Kern and Meir, 2001).

*Fractional Flow Reserve*

Another method for assessing the physiologic severity of an intermediate coronary lesion is the fractional flow reserve (FFR). The method is based upon the calculation of the pressure within the wire during maximum myocardial blood flow in the presence of a stenotic supplying coronary artery, divided by the normal maximal flow in the same distribution. The calculation of this flow index is derived from the ratio of the mean distal coronary artery pressure to the mean aortic pressure during hyperemic vasodilation. First, the pressure wire is calibrated to zero, then it is advanced across the lesion under investigation, and adenosine (18 to 40μg bolus) is administered directly

into the ostium of the coronary artery through the guiding catheter to induce maximal CBF. Subsequently, the distal coronary artery pressure is measured and the FFR is calculated using the previously stated formula. The normal value of the FFR is 1.0. An index of 0.75 to 0.90 indicates a lesion of intermediate significance and values less than 0.75 indicate a pathological stenosis (Kini, 2006, Pijls et al., 1996).

### 1.7.2 Coronary Stents

Historically, abrupt vessel closure was treated by emergency coronary artery bypass surgery (CABG). However, surgery under these conditions was associated with an increased mortality. Even with the introduction of perfusion catheters which allowed prolonged balloon inflation with simultaneous tissue perfusion through the balloon catheter's central lumen, the success rate of treating acute vessel closure remained low. Fortunately, coronary stents, with their scaffolding properties, were brought into practice to control abrupt closure temporarily until bypass surgery could be performed (Ozaki and Serruys, 1997; Serruys et al., 1999). Coronary stents are metallic mesh conduits which are implanted over the area of stenosis and deployed by balloon inflation to function as a structure to maintain the lumen of the vessel wall with the expectation of circumventing abrupt closure and restenosis (Schatz, 1988; Hara et al., 2006). Intravascular ultrasound studies have demonstrated that over the long term, stents do not recoil (Hoffman et al., 1996). Consequently, negative remodelling from stent implantation is removed, and as a result in-stent restenosis is mainly due to neointimal proliferation. Previously, intra-coronary stents were indicated for: (1) the prevention of coronary restenosis; (2) the treatment of an acute myocardial infarction; (3) the treatment of saphenous vein graft stenosis; and (4) the prevention of acute vessel closure (Lane, 1999). Coronary stents have been utilized in over 85% of PCIs since the late

1990s because of the decreased restenosis rate compared to balloon angioplasty and other newer interventional catheter-based devices (Lane, 1999; Kini, 2006). Clinical in-stent restenosis (ISR) remains a major problem with bare metal stents, occurring in 30-40% of patients following PCI (Erbel et al., 1996; Cutlip et al., 2002). There are two basic types of stents: bare metal (BMS: uncoated stents) and drug eluting stents (DES).

Bare Metal Stents and Thrombosis

Stent thrombosis is the result of intimal injury to the vessel during PCI in which the release of procoagulant tissue factor and the exposure of collagen and adhesion molecules stimulate the production of a platelet-rich clot that seals the site of injury. The degree of platelet deposition and thrombus formation can lead to myocardial ischemia, injury and possibly necrosis (Van Belle et al., 2007; Lam et al., 1986). BMS studies in animals and humans have revealed that reformation of an intact layer of endothelium is achieved after about one month post-BMS implantation (Anderson et al., 1992; van Beusekom et al., 1993). An intact endothelium is critical to circumventing mural thrombus formation and the lack of which is the primary cause for the risk of thrombosis seen during the first month post-PCI (Van Belle et al., 2007). In the developmental stages of PCI, stent thrombosis reached as high as 12% despite heavy antithrombotic treatment mixing heparin, coumadin, asprin (ASA) and other anticoagualants. It took ten years after the initial stent implantations to discover that dual antiplatelet therapy (asprin and ticlopidine) was the key in reducing the stent thrombosis rate to less than 1%. To date clopidogrel (loading dose 300 mg followed by 100mg/day), asprin (100mg/day), and statins are the standard treatment following PCI with a BMS (Serruys et al., 1999; Serruys et al., 2002). The treatment is given for one

month and thereafter the clopidogrel is discontinued and the patient remains on asprin indefinitely (Leon et al., 1998; Bertrand et al., 1998).

## Drug-Eluting Stents

Drug-eluting stents are impregnated with a polymeric drug coating that has anti-inflammatory and anti-proliferative actions through continuous local release (Schwartz et al., 2004). Drug-eluting stents have been demonstrated to attenuate neointimal hyperplasia significantly in animal models and to decrease the rate of restenosis in humans (Suzuki et al., 2001; Grube et al., 2007). Some apprehension has been fostered concerning delayed endothelialization of the coated prothesis causing late stent thrombosis appearing primarily when antiplatelet therapy is stopped in the follow-up period (Van Belle et al., 2007). The two major drug-eluting stents, the Taxus (Boston Scientific), and the Cypher (Johnson and Johnson), are coated with paclitaxel and sirolimus, respectively (Herrmann, 2003; Goy et al., 2005).

### *Paclitaxel-Eluting Stents*

Paclitaxel is a drug which decreases cell division and migration through inhibition of microtubule formation (Herrmann, 2003). Studies have been conducted on the release formulations of paclitaxel-eluting stents compared to bare metal stents in humans with low-risk coronary lesions undergoing PCI. The TAXUS II trial used two different paclitaxel release formulations to show the safety and efficacy in a large group of patients. The stents were coated with a polymer designed to control paclitaxel release with a fundamental burst phase over the initial 48 hours following implantation, then a low-level release for ten days. The low-level release formulation was either slow release, where 90% of the total dose loaded (paclitaxel eluting stents were coated with a dose of 1 $\mu g/mm^2$) remained sequestered within the polymer formulation, or moderate release

where 75% remained sequestered within the polymer formulation. Coronary angiography after six months revealed that the biphasic polymer-managed delivery of low levels of paclitaxel produced substantial decreases in neointimal proliferation as compared to controls (Columbo et al., 2003). However, other studies measuring the effects of paclitaxel delivery using different dosing and delivery systems revealed that the ability to deliver large quantities of paclitaxel through acrylate polymer coatings was correlated with high incidences of acute, subacute, and late in-stent thrombosis (SCORE Trial; Sousa et al., 2003). In addition, paclitaxel release from stents with nonpolymer-based delivery systems did not translate into patient clinical benefit (Parks et al., 2003). The patients in the TAXUS II trial had low risk lesions. It is not clear whether a slow-release formula would be applicable to high risk patients or patients who present with complex lesions. The attenuation of restenosis has been demonstrated with drug-eluting stents in large arteries with short lesions. However, there is not a substantial body of evidence demonstrating a decrease in the frequency of clinical restenosis in patients with disease bifurcation, chronic total occlusions, saphenous vein graft disease, or multivessel disease (Moses et al., 2003).

*Sirolimus-Eluting Stents*

Sirolimus (rapamycin), a naturally occurring product that was discovered in a soil sample from Easter Island, is isolated from the species Streptomyces hygroscopicus. It is a lipophilic macrolide that has a potent immunosuppressive effect on mammals, and has an impact on the care of patients with coronary artery disease because of its ability to inhibit vascular smooth muscle proliferation, and thus prevent ISR (Marx et al., 1995; Poon et al., 1996). The process by which sirolimus retards cell growth is related to cell cycle arrest at the transition from the $G_1$ to S phases (Poon et al., 1996; Luo et al., 1996).

A study in pig models revealed that systemic sirolimus administration could inhibit restenosis in a model of angioplasty (Gallo et al., 1998). The Sirolimus-Eluting Bx Velocity Balloon-Expandable Stent utilized in a randomized study (RAVEL), attenuated the frequency of ISR (Morice et al., 2002; Fajadet et al., 2005; Windeker et al., 2005). The SIRIUS trial evaluated the frequency of ISR among patients with complex coronary artery disease who had sirolimus-coated stents implanted, and found that the restenosis rate decreased to 18% in the sirolimus group compared to a 51% restenosis rate in the bare metal stent group (Lemos et al., 2003; Schampaert et al., 2004; Schofer et al., 2003). However, it is not clear as to the duration of inhibitory effects induced by sirolimus, or whether or not there are subgroups of patients who are more resistant than others to therapeutic consequences of sirolimus (Schwartz et al., 2004; Moses et al., 2003). DES has become the treatment of choice for ISR following BMS implantation. Two recent prospective, multicenter, randomized, clinical trials have demonstrated that DES had better clinical and angiographic outcomes when compared with vascular brachytherapy for the treatment of BMS-related ISR (Stone et al., 2006).

Drug-Eluting Stents and Thrombosis

The RAVEL trial and several other randomized trials clearly demonstrated a significant reduction in the restenosis rate in patients with DES implantation (Morice et al., 2002; Fajadet et al., 2005; Windeker et al., 2005). However, there are concerns about residual deposits of fibrin and retarded endothelialization on DES pathological specimes (Moreno et al., 2005). The first clinical indication of thrombosis was reported by McFadden et al (2004), in which four cases of late DES thrombosis occurred, primarily after antiplatelet discontinuation. Data presented at the Transcatheter Therapy (TCT) Meeting in Washington, D.C. USA, 2006 showed that there was no difference between

DES and BMS with respect to cardiac, non cardiac and total mortality or MI at 3-4 year follow-up. In addition, there was a small significant difference (p=0.048) in stent thrombosis between DES and BMS when considering the data beyond one-year follow-up (Van Belle et al., 2007). The Academic Research Consortium (ARC) came to an agreement on two classes of stent thrombosis: (1) Definite/confirmed stent thrombosis occurs during acute coronary syndrome with either angiographic or autopsy evidence or thrombolytics in myocardial infarction (TIMI) flow grade 0 with occlusion originating in the stent or at a 5 mm segment proximal or distal to the stent and (2) Probable stent thrombosis which includes all cardiac deaths occuring within 30 day post-PCI and MI occuring within the area suplied by the targeted stented vessel (with or without angiographic confirmation) (Applegate et al., 2008). Stent thrombosis continues to be a major complication with DES. The risk of late stent thrombosis is related to slow endothelialization. However the rate of DES stent thrombosis is close to that of BMS at 4-year follow-up. Thus, dual antiplatelet therapy is recommended. BMS implantation is still recommended for the following circumstances (1) vessels greater than 3.5 mm, (2) patients incapable of taking their dual antiplatelet medication faithfully, (3) patients who may need another significant intervention; and (4) the high cost of DES (Van Belle et al., 2007)

**1.8 PCI and Coronary Restenosis**

In 2006 there were more than 900,000 PCIs performed in North America, many of these repeat procedures on the same patients (Larose, 2006). Restenosis is an adverse outcome following balloon angioplasty and stenting for stenosis, in which there is a re-narrowing of the artery. The high incidence of restenosis following PCI progressively limits long-term benefits of the procedure. Restenosis is most likely the result of

physical damage to the endothelial and subintimal layers of the vessel from the PCI procedure itself causing constrictive vessel remodeling. Consequently, the artery attempts to repair itself through the stimulation of smooth muscle cells to migrate from the media of the vessel wall into the subintimal region where they proliferate (Serruys et al., 1994; Sigwart et al., 1987). SMC proliferation effectively results in neointimal hyperplasia, and vascular remodeling that may eventually hinder adequate blood flow because of lumen size reduction. This ultimately induces major cardiac events (MACE) i.e. myocardial ischemia, infarction, thrombosis, or death (Serruys et al., 1991; Serruys et al., 1994; Sigwart et al., 1987). The two major categories of restenosis for balloon angioplasty and stent implantation are angiographic restenosis, defined as a less than 50% reduction of lumen diameter at follow-up angiography, which occurs in 25 to 50% of all cases, and clinical restenosis, defined as recurrent angina (chest discomfort) with angiographic restenosis greater than 50% which occurs in 25 to 45% of patients (Hansrani et al., 2002). Clinical restenosis usually occurs within the first six to nine months following the procedure (Hansrani et al., 2002).

In-Stent Restenosis (ISR)

Winslow et al. (2005) demonstrated that the damage caused by inflation of the balloon-tipped catheter system and deployment of the metal stent (PCI) initiates ISR. PCI-induced ISR is essentially attributed to neointimal hyperplasia of smooth muscle cells, and to a limited degree of mural thrombosis (Ott et al., 1998; Kraemer, 2000; Carter et al., 1996; Frimerman et al., 1997; Liu et al., 1989). Aronson (2002) described the initial response to stenting as the accumulation of a layer of thrombus forming over stent struts, followed by an accelerated conglomeration of inflammatory infiltrate (Ishiwata et al., 1997; Schober and Weber, 2005; Montalescot et al., 1995). Scott (2006) reported that

three days post-stent implantation there was an accumulation of neutrophils in the stent struts and they were related to the underlying arterial wall morphology. Studies conducted by Farb et al. (1999), and Komatsu et al. (1998), showed a positive correlation between the extent of inflammation and the degree of ensuing neointimal development. PCI causes endothelial damage in which macrophages and polymorphonuclear neutrophils are recruited to the area of endothelial dysfunction through chemotaxis and endothelial expression of adhesion molecules (Schulze et al., 2002; Furukawa et al., 1999; Grewe et al., 2000). Leukocytes transmigrate into the subendothelium where they release cytokines (TNF-α) and growth factors that induce the secretion of matrix metalloproteinases. Metalloproteinases are macromolecules that contribute to remodeling of the extracellular matrix through SMC migration to the focus of the developing restenotic lesion (Southgate et al., 1996; Huber et al., 1992). Moreno et al. (1996), studied unstable angina patients who underwent PCI (atherectomy), and upon follow-up angiography, identified the patients who developed post-PCI restenosis. The restenosis group and the non-restenosis group both had tissue biopsies of the areas of previous PCI. Immunostaining of the coronary samples revealed that those patients who developed restenosis had a significantly higher concentration of macrophages in their lesions compared to those patients who did not develop restenosis. This study implies that macrophage content is positively correlated with post-PCI restenosis in patients with unstable angina.

According to Kastrati et al. (2001), one of five patients who receive bare metal stent implantations will develop ISR. The available treatment modalities for ISR are repeat PTCA, directional atherectomy, brachytherapy, DES, and repeat implantation of a BMS. Using a porcine model to study ISR, McKenna et al. (1998) have shown that the

neointimal hyperplasia associated with ISR is more effectively treated with rotational atherectomy (RA) as opposed to PTCA. On the other hand, in the Angioplasty vs. Rotational Atherectomy for the Treatment of Diffuse In-Stent Restenosis Trial (ARTIST) in which 298 patients randomly received PTCA or RA with adjunctive PTCA when restenosis was identified at post-PCI follow up, there was found to be no difference in procedural success. However, at 12 months post-PCI, patients who were treated with PTCA at six months had lower restenosis and MACE rates compared to those patients who received RA (vom Dahl et al., 2002). Adding to the controversy, the Rotational Atherectomy vs. Balloon Angioplasty for Diffuse In-stent Restenosis trial (ROSTER) demonstrated that at a mean follow-up of 12 months, target lesion revascularization (TLR) rates were lower, and there was less residual hyperplasia measured by intravascular ultrasound (IVUS) for patients treated with RA compared to PTCA (Scharma et al., 2004). The recent debatable data pertaining to the effectiveness of RA and the treatment of ISR has become overshadowed with the advent of the drug-eluting stent (Tran et al., 2008).

Pathology of ISR

The complex healing that occurs after stenting involves the following overlapping mechanisms: (1) elastic recoil (vasoconstriction) in response endothelial disruption, occurs within 24 hours; (2) mural thrombus formation from local platelet activation and thrombin secretion occurs within two to three weeks; (3) neointimal hyperplasia (major factor): this occurs when smooth muscle cells grow and replicate to produce an extensive volume of extracellular matrix over a period of 2 days to months; (4) remodelling process: a change in artery wall size, without a change in artery wall mass.

The overriding mechanism that contributes to restenosis is inflammation which is found at all stages of restenosis (Lafont et al., 1995; Anderson et al., 1996).

ISR and Inflammation

Stenting leads to injury in the arterial wall which is evidenced by acute inflammation with resultant infiltration of monocytes from the lumen into the developing thrombus. Monocytes secrete enzymes which change the composition and structure of the thrombus. They also transmigrate into the subendothelium and differentiate into macrophages capable of becoming foam cells at the focus of the newly developed lesion. IL-1, TNF-α and AGE induce the production of growth factors such as fibroblast growth factor which plays a role in plaque progression. Injury to the endothelium stimulates an inflammatory reaction, and proliferative growth in the media. Afterwards, vascular smooth muscle cells migrate into the intimal region where they increase extracellular matrix by proliferation, thereby adding bulk to the restenostic lesion. Interestingly, vascular smooth muscle cells undergo morphological changes promoting a loss of their contractile characteristics causing them to become synthetic cells. The two phases of restenosis are: (1) the early phase: controlled by inflammation; and (2) the late phase consisting of multiple mechanisms promoting increased lesion volume. Finally, the steps in both phases are controlled by interrelated molecular and cellular interactions among AGEs, RAGEs cytokines, growth factors, secondary messengers, and proto-oncogenes involved in the transcription, translation, and post-translational occurrences (Kibos et al., 2007; Ross, 1995; Farb et al., 1999; Komatsu et al., 1998).

### 1.8.1 Biochemicals Features of Restenosis

*Oxygen Radicals and Restenosis*

The endothelial injury induced by the PCI itself can activate platelets and neutrophils that can produce ROS. Reactive oxygen species formed at the site of damage can stimulate chain reactions of endothelial dysfunction and LDL oxidation leading to restenosis. OxLDLs are engulfed by monocytes which become macrophages which in turn liberate numerous growth factors that induce SMC proliferation. SMC proliferation, migration, and collagen accumulation are major determinants of neointimal growth. Mediators of SMC proliferation include ROS (Teirstein and King, 2003; Marumo et al., 1997; Sundaresen et al., 1995). SMC proliferation and stenosis after vascular injury coincide with elevations in ROS (Szocs et al., 2002; Shi et al., 2001). Reactive oxygen species have been implicated in growth signaling pathways and in SMC proliferation (Colavitti et al., 2002; Abe and Berk, 1999; Bhunia et al., 1997). Jacobson et al. (2003) have indicated that superoxide anions are involved in neointimal development after vessel injury, based on the administration of the neointimal inhibitor gpg1ds-tat (the chimeric peptide inhibitor of the NADPH oxidase assembly). Moreover, ROS have been implicated in the development of hypercholesterolemic atherosclerosis (Gupta and Prasad, 1992; Prasad and Kalra, 1993; Prasad, 1999; Prasad et al., 1994; Prasad, 2005; Prasad, 1997b). Tardif et al. (1997) investigated the oxidative stress produced by PCI with subsequent restenosis. In a double blind randomized trial, they studied whether drugs with antioxidant properties attenuated the occurrence and severity of restenosis following angioplasty. Their work suggested that antioxidants may have

inhibited endothelial dysfunction, LDL oxidation, and attenuated neointimal formation, all of which may be involved in the mechanism of restenosis (Tardif et al., 2003).

The sources of oxygen radicals during PCI could be numerous, including xanthine-xanthine oxidase, mitochondria, polymorphonuclear leukocytes, arachidonic acid metabolites, AGE/RAGE activation and CRP (Prasad and Bharadwaj, 1996; Prasad et al., 1996; McCord and Roy, 1982; Prasad, 1999; Misra, 1974; Kanellakis et al., 2004; Bierhaus et al., 2005; Bierhaus et al., 1997a; Bierhaus et al., 1997b; Prasad, 2006b; Aronson, 2002, Zhou et al., 2003; Crapo, 1986). Thus, it is plausible that ORs may be involved in restenosis following PCI (McNair et al., 2006).

*Inflammatory Mediators and Restenosis*

A few of the pleiotropic effects of cytokines produced by endothelial cells, vascular SMC, and circulating lymphocytes are the induction of cellular inflammation, cell proliferation and apoptosis in cardiovascular disease (Neumann et al., 1995). As summarized by Hojo et al. (2001), the concentrations of cytokines, particularly IL-6 and IL-8, are increased in acute coronary syndromes. Studies conducted by Serrano et al. (1997) suggested that PCI stimulates inflammatory responses and increases the expression of cytokines. Libby et al. (1992) described a "cytokine cascade" in which the PCI-induced local inflammation and release of cytokines triggers the further release of vasoactive substances, thrombin production and activation of growth factors which can ultimately lead to restenosis (Libby and Galis, 1995). Plasma levels of endothelial-derived M-CSF (hemapoietic growth factor) are increased in patients with unstable angina and in those with acute myocardial infarction. It was also shown that the levels of the anti-inflammatory cytokine, transforming growth factor beta (TGF-β) in patients

with unstable angina were decreased to levels lower than controls, and that the ratio of the plasma concentrations of MCS-F and those of TGF-β is related to the degree of coronary atherosclerosis (Tashiro et al., 1995; Tashiro et al., 1997). The chemokines [CCL-8 (IL-8) and CCL-2 (MCP-1)] and M-CSF have been implicated in the development of restenosis post-PCI because of their ability to stimulate migration and activation of neutrophils, activation of adhesion molecules on the endothelium, as well as proliferation and differentiation of monocyte/macrophages (Hojo et al., 2001). In another study Tashiro et al. (2001), indicated that post-PCI levels of plasma M-CSF in patients who developed restenosis were elevated compared to those patients who did not develop restenosis.

The pathophysiological genesis of restenosis may be similar to the genesis of atherosclerosis because both could possibly be attributed to endothelial injury, although the sources of endothelial damage are different. The endothelial injury associated with restenosis is not due to the common risk factors for atherosclerosis (hyperlipidemia, family history, smoking status, etc.), but can be attributed to injury from intra-luminal radial forces created by inflation of intra-coronary balloons and deployment of the metallic stents alone (Kuntz and Baim, 1993; Winslow et al., 2005). The PCI-induced vascular injury may stimulate a confined inflammatory response, activating the endothelium with expression of adhesion molecules and release of chemokines which orchestrate the recruitment of circulating inflammatory cells (Cipollone et al., 2001). The vascular remodeling and local inflammatory activity may be due to the liberation of local and systemic cytokines (Hayashi et al., 2000; Tashiro et al., 2001). It has been demonstrated that there is systemic inflammatory stimulation and increased expression

of vascular adhesion molecules in patients with angina pectoris and acute myocardial infarction. Therefore, regional vascular inflammation and tissue damage may be a reaction to systemic proinflammatory mediators (Cipollone et al., 2001; Hayashi et al., 2000). Schulze et al. (2002) reported that serum cytokine levels of IL-1β increased significantly at 48 hours from baseline values in patients who underwent PCI and later developed restenosis. Conversely, those patients without restenosis showed a decrease in IL-1β at 48 hours. In addition, serum levels of the adhesion molecules ICAM-1 and VCAM-1 decreased significantly at 48 hours from baseline in those patients who did not develop post-PCI restenosis. Their work revealed the existence of periprocedural variations in serum levels of cytokines and adhesion molecule expression within 48 hours of PCI, lending support to the speculation that inflammatory mediators are involved in restenosis and may be biochemical markers for this phenomenon. Moreover, Pietersma et al. (1995) investigated whether plasma granulocytes and monocytes could be used as pre-procedural biochemical markers/ predictors of post-PCI restenosis. Since activated granulocytes express high levels of integrins that are involved in the ligand counterreceptor mechanisms associated with their adhesion to the endothelium, they measured plasma levels of CD64, CD66 and CD67. In addition, they measured plasma levels of IL-1β1 and TNF-α, proinflammatory mediators that indicate an active state of monocytes. Ultimately, their goal was to detemine whether lumen-renarrowing post-PCI was correlated with stimulation of circulating phagocytes. Their results showed a significant inverse relationship between pre-procedural plasma levels of CD66 and lumen renarrowing at the six month follow-up PCI. These data imply that granulocytes may have a protective effect against restenosis. In addition, their results demonstrated a positive correlation between the pre-procedural plasma levels of IL-1β

and renarrowing of the vessel lumen. This suggests that activated monocytes may contribute to post-PCI restenosis. Finally, their work also demonstrated that there were no significant differences of TNF-α between the pre-procedural levels and the six month post-PCI plasma levels in either the restenosis or non-restenosis group (Pietersma et al., 1995).

#### 1.8.1.1 sVCAM-1 and Restenosis

Atherosclerosis and restenosis are primarily inflammatory reactions at the site of vascular injury. Both are distinguished by the infiltration of leukocytes into the foci of inflammation (Butcher, 1991; Springer, 1994). The recruited leukocytes are mainly monocytes that migrate into the activated endothelium under the influence of adhesion molecules such as VCAM-1 (Heider et al., 2006). VCAM-1 and ICAM-1 have been detected on the intimal SMC in the atherosclerotic vascular wall (Jang et al., 1994). The soluble form (sVCAM) liberated from endothelial cells, platelets and SMC, can be identified in the serum (Miwa et al., 1997). Miwa et al. (1997) demonstrated elevated levels of sVCAM-1 and sICAM-1 in the systemic and coronary circulation of patients with variant angina. In addition, Pigott et al. (1992) showed that the concentration of sVCAM-1 reflects the increased expression of VCAM-1 on the endothelial cell surface. Early reports concerning the expression of adhesion molecules on smooth muscle and endothelial cells in restenosis were derived from animal studies in rabbit aortas. Balloon injury and resultant endothelial denudation of rabbit aortas induced ICAM-1 expression on endothelial cells two days post-trauma. The same procedure produced an increased expression of ICAM-1 and VCAM-1 in neointimal SMC at 5 to 10 days which remained constant for at least 30 days after injury (Tanaka et al., 1993; Tanaka et al., 1995a;

Tanaka et al., 1995b). In addition, Manka et al. (1999) described the increased expression of VCAM-1 in the carotid arteries of apolipoprotein-E-deficient mice. During restenosis, medial SMC experience a phenotypic change from a silent contractile to a synthetic cell type similar to fetal smooth muscle cells (Ardehali et al., 1995; Owens, 1995). These SMC then proliferate and migrate from the media to the intima of adjacent arterial walls where they begin to divide and generate extracellular matrix, resulting in the production of intimal lesions (Peterson et al., 2008). Cytokine-stimulated SMC migration is regulated by the expression of VCAM-1, which interacts with the actin cytoskeleton and the extracellular matrix (Moiseeva, 2001). Currently, studies on the mouse injury model have shown that VLA-4 (counterreceptor for VCAM-1) is present on SMC, and that VCAM-1 is necessary for the accumulation of SMC in the neointima (Barringhaus et al., 2004; Dansky et al., 2001). Conversely, inhibition of the VCAM-1/VLA-4 pathway attenuates monocyte adhesion, infiltration and neointimal generation following arterial injury (Oguchi et al., 2000; Barringhaus et al., 2004). Peterson et al. (2008) studied SMC isolated from the aortas of B6 mice which were susceptible to diet-induced atherosclerosis. They investigated whether SMC express VCAM-1 and whether it aids in migration. Essentially, SMC migration in a specified direction requires a cell extension of anterior llamelopodia that connect to a substrate, and then contract. Next, there is detachment at the posterior end of the cell, permitting tail retraction and forward translocation of the cell body. The capacity to spread and migrate seems to be reliant upon a particular adhesive strength between cell and substrate: elevated or lowered degrees of substrate attachment attenuate spread and migration. On the other hand, intermediary adhesion strengths allow for maximum migration (Ridley et al., 2003; Lauffenburger and Horwitz, 1996; Palecek et al., 1997).

Furthermore, Peterson et al. (2008) suggested that the VLA-4/VCAM-1 interaction increases the proliferation of SMC in response to stimuli such as collagen and/or fibronectin (Nguyen et al., 2005). However, the mechanism of action is unclear at this time. These data imply that SMC express VCAM-1 which aids in cell migration. It has been demonstrated that SMC migration is an essential step in post-PCI restenosis. Smooth muscle cell migration and proliferation are the hallmarks of post-PCI restenosis. Thus, therapeutic inhibition of sVCAM-1 may play a role in the prevention of this phenomenon.

1.8.1.2 TNF-alpha and Restenosis

A novel study conducted by Bose et al. (2007) utilized a distal balloon protection device during PCI in order to examine the presence of TNF-α in saphenous vein grafts (SVG) The distal balloon protection device (BPD) captures and aspirates particulate matter and soluble material during stent implantation. They investigated whether TNF-α was liberated into the coronary circulation (which could be aspirated by the BPD), and whether this release was related to changes in plaque volume and the development of post-PCI restenosis at five months follow-up. Their findings demonstrated that patients who developed post-PCI restenosis with a decrease in lumen diameter of greater than 50% had significantly higher TNF-α levels after PCI as compared to those patients who developed post-PCI restenosis with lumen diameters of less than 50%. In addition, their results showed that TNF-α was liberated into the aspirate of the stented SVG and that this discharge was correlated to the reduction of plaque volume and restenosis. This evidence suggests that the peri-procedural concentration of TNF-α at the site of the stented SVG lesion may predict the level of restenosis at five months post-PCI. Takeda

et al. (2005) reported that endogenous cytokines, particularly TNF-α, are involved in the increased expression of RAGE in the endothelium and in neointima in the femoral artery of obese Zucker rats. This data represents a link between TNF-α, RAGE and the neointimal hyperplasia associated with restenosis after injury.

1.8.1.3 CRP and Restenosis

C- reactive protein (CRP), a known marker of inflammation and predictor of risk of the development of atherosclerosis, stable and unstable angina and acute myocardial infarction (Ridker and Silvertown, 2008; Ridkeret al., 1998b; Prasad, 2006b; Meuwissen et al., 2006), has been studied in relation to post-PCI restenosis. Tashiro et al. (2001) reported that the pre-PCI plasma concentrations of CRP were not significantly different in patients who did or who did not develop restenosis. However, the post-PCI levels of plasma CRP were notably lower in patients who did not develop post-PCI restenosis compared to those who developed restenosis. Accordingly, inflammatory mediators may be implicated in the pathogenesis of coronary restenosis and their levels may be useful indicators for anticipating the likelihood of this adverse outcome.

As detailed earlier, restenosis may be in part the result of neointimal formation in the artery after injury from the PCI. According to Schwartz (1997), the early consequence of post-PCI endothelial injury in rats is smooth muscle cell proliferation. He also concluded that the initial wave of proliferation from the media is associated with SMC formation in the intima. Moreover, he suggested that low but continuous migration from the media is regulated by integrins, platelet derived grown factor (PDGF), angiotensin II, transforming growth factor beta (TGF-beta) and fibroblast growth factor (FGF).

According to Caixeta et al. (2007), proinflammatory cytokines and inflammatory markers are secreted into the systemic circulation early post-PCI, and levels of CRP are positively correlated with clinically relevant restenosis. CRP is a mediator of atherosclerosis by inducing arterial activation and macrophage recruitment. In addition, CRP may also contribute to inflammation and restenosis through RAGE activation. Zhong et al. (2007) have suggested that CRP is a RAGE ligand which can attach to RAGE and induce cellular activation. These data suggest an association between CRP, RAGE, atherosclerosis, and restenosis post-PCI.

Finally, the release and/or expression of cytokines, adhesion molecules, matrix metalloproteinases, growth factors and other related inflammatory molecules all might play a role individually or in concert to induce the genesis and/or progression of post-PCI restenosis.

1.8.1.4 Advanced Glycation End Products, Atherosclerosis and Restenosis

The interaction of AGE with RAGE promotes stimulation of the ROS-sensitive transcription protein NF-κB resulting in the following: (1) increased expression of cytokines; (2) increased expression of adhesion molecules; (3) increased expression of growth factors; (4) increased expression of tissue factor; and (5) the generation of ROS (Bierhaus et al., 2005; Bierhaus et al., 1997a; Bierhaus et al., 1997b; Prasad, 2007; Aronson, 2002). AGE binding to RAGE has been associated with the pathogenesis of atherosclerosis, coronary artery disease, coronary restenosis, hypertension (HTN), and diabetic vasculopathies (Brownlee et al., 1988; Bierhaus et al., 1998; Bierhaus et al., 2005; Bierhaus et al., 1997a; Bierhaus et al., 1997b; Prasad, 2006; Aronson, 2002; Park et al., 2001; Zhou et al., 2003; Sakaguchi et al., 2003). The vascular disease associated

with the AGE-RAGE interaction has been well documented in diabetic and non-diabetic atherosclerosis. The three classes of RAGE ligands which can stimulate the receptor-mediated cellular activation are: (1) the products of nonenzymatic glycoxidation (AGEs); (2) s100 proteins (calcium binding proteins found in inflammatory lesions); and (3) amphoterin (nuclear protein released from necrotic or damaged cells) (Basta, 2008; Hori et al., 1995). Given the diversity of these endogenous ligands implies that RAGE is a unique sensor for environmental signals and may play a critical role in the regulation of homeostasis and pathogenesis (Basta, 2008). AGEs are abundant on long-lived vessel wall proteins, and are present in the intima, media, and adventitia at sites of atherosclerotic lesions. The vascular ligands of RAGE are characterized by their ability to fluoresce and/ or cross-link proteins. The cross-linking of proteins reduces their flexibility, elasticity, and functionality. The three categories of AGEs are the following: (1) fluorescent cross-linking species (pentosidine and crossline); (2) non–fluorescent cross-linking species (arginine-lysine imidazole); and (3) non-cross-linking species (pyrraline and carboxymethyl lysine [CML]) (Niwa et al., 1997). AGE formation is accelerated under conditions of oxidative stress such as the pro-oxidant environment of lipid-rich vascular lesions (Nishikawa et al., 2000). The nonenzymatic cross-linking of proteins by AGEs can initiate deleterious inflammatory reactions such as the signalling of proinflammatory cytokines, extracellular matrix expansion, and production of growth factors (Bierhaus et al., 1998). According to Bucala and Cerami (1992) AGEs are found with circulating low-density lipoprotein. CML forms *in vitro* from LDL incubated with copper ions and glucose that suggests that it is both a protein and lipid adduct. In addition, activated neutrophils can produce CML on proteins by a myeloperoxidase-dependent pathway. The various pathways of CML formation imply that there is a

direct link between inflammation and AGE production. Iwashima et al. (2000) reported that the expression of oxidized LDL receptors on human monocytes that are eventually converted to macrophages (by M-CSF) can be influenced by AGEs. Specifically, the enhanced expression of oxidized LDL receptors on macrophages was positively correlated with uptake of modified LDL and increased foam cell formation. Foam cell formation leads to the genesis of the early atherosclerotic streak. In a recent study, it was shown that treatment of human macrophages with AGEs resulted in an attenuation of the mRNA and protein levels in the ATP-binding cassette transporter G1 (causes the efflux of cholesterol to bigger HDL molecules) in a RAGE-dependent manner (Isoda et al., 2007). This implies that AGEs may be involved in the genesis of atherosclerosis by foam cell formation via the increased quantities of oxLDL receptors and via lowered cholesterol efflux to HDL. In addition, the RAGE-ligands may amplify the inflammatory responses via increased production of proinflammatory adhesion molecules, cytokines and metalloproteinases. Moreover, the AGE-RAGE interaction may underlie ongoing amplification of inflammatory events in tissues previously sensitized by the deposition of lipids or by immune/inflammatory stimuli (Basta, 2008).

The association of the AGE-RAGE interaction and the development of restenosis have been demonstrated in diabetic rats undergoing carotid artery injury induced by balloon angioplasty and denudation (Zhou et al., 2003). Following denudation, there was a significant accumulation of AGEs (CML and S100 proteins) in the arterial wall with upregulation of RAGE, and the development of neointimal proliferation. However, blockade of RAGE or the administration of sRAGE resulted in a significantly attenuated neointimal expansion after arterial injury and decreased SMC proliferation, migration,

and expression of extracellular matrix proteins (Park et al., 2001; Zhou et al., 2003; Sakaguchi et al., 2003).

1.8.1.5 sRAGE and Atherosclerotic Plaque Vulnerability

The atherosclerotic plaque rupture and thrombosis of patients suffering from myocardial infarctions is due primarily to an inflammatory process which destabilizes the fibrous cap tissue of the lesion independent of plaque morphology (van der Wal et al., 1994; van der Wal, 2007). Diabetic atherosclerotic plaque macrophages detected in human aortas have been shown to have an increased expression of RAGE which is linked to augmented inflammatory reactions and cyclooxygenase-2 (Cox-2) and microsomal prostaglandin E synthetase-1 (mPGES-1) expression (Kume et al., 1995). Cipollone et al. (2003) demonstrated an association between RAGE inflammatory overexpression and increased Cox-2 expression in plaque macrophages and this effect may induce plaque destabilization by increasing MMP expression. RAGE-induced proinflammatory mediators accounted for sustained increases in vascular expression of VCAM-1, Cox-2 and MMP-9 antigen and activity in the aortas of diabetic apoE-deficient mice (Bucciarelli et al., 2002). However, administration of sRAGE significantly suppressed the levels of MMP-9 antigen and activity in diabetic mice aortas (Bucciarelli et al., 2002; Wendt et al., 2005). Taken all together, these data imply that blockade of the function of RAGE with sRAGE may avert the destabilization of susceptible plaque associated with acute coronary syndromes.

1.8.1.6 sRAGE and Suppression of Restenosis

The C-truncated isoform of sRAGE is proteolytically cleaved by extracellular matrix metalloproteinases from native membranous receptors (Hudson et al., 2005). The

interaction of AGE and RAGE has been shown to play a role in the development and progression of coronary artery stenosis (Bierhaus et al., 2005). In human and animal models, coronary stent implantation has been demonstrated to increase circulating levels of AGE adducts such as n-ε-(carboxymethyl) lysine (CML) (Sakaguchi et al., 2003; Basta et al., 2008a; Basta et al., 2008b). sRAGE suppresses neointimal growth following arterial denudation by sequestering RAGE ligands and preventing their interaction with cell receptors (Yonekura et al., 2003). Zhou et al (2003) reported that the levels of AGEs and RAGE in the carotid arterial endothelium were higher in Zucker diabetic rats compared to euglycemic controls. They induced injury to the carotid arteries in these rats by balloon inflation which further increased the concentrations of AGEs and RAGEs, and produced in neointimal hyperplasia. However, treatment with sRAGE before, and up to 21 days post-balloon damage, significantly attenuated the neointimal growth. Likewise, Sakaguchi et al. (2003) demonstrated that arterial endothelial denudation in wild type mice up-regulated RAGE in injured vessels, specifically in smooth muscle, and increased AGE accumulation in developing neointima. The administration of sRAGE lowered neointimal expansion, SMC proliferation and migration and expression of extracellular matrix proteins. Neointimal expansion in RAGE -/- mice was suppressed, compared with that detected in wild type littermates using a femoral artery denudation protocol to induce arterial injury (Sakaguchi et al., 2003). AGEs activate the growth factor of microvascular endothelial cells by inducing vascular endothelial growth factor. This leads to angiogenesis, the inhibition of prostacyclin production, and the stimulation of plasminogen activator inhibitor-1 synthesis by endothelial cells, thus inducing thrombogenesis (Sakaguchi et al., 2003; Yamagishi et al., 1997; Yamagishi et al., 1998; Basta et al., 2008b). These

data suggest that sRAGE administration may be an effective treatment in the prevention and treatment of post-PCI restenosis. AGE-RAGE interaction in the presence of high levels of serum sRAGE would favor prevention of atherosclerosis/restenosis. However, AGE-RAGE interaction with lowered levels of serum sRAGE would favor atherosclerosis/ restenosis (Figure 1).

AGE/sRAGE Ratio Determines Either Stenosis or Cytoprotection

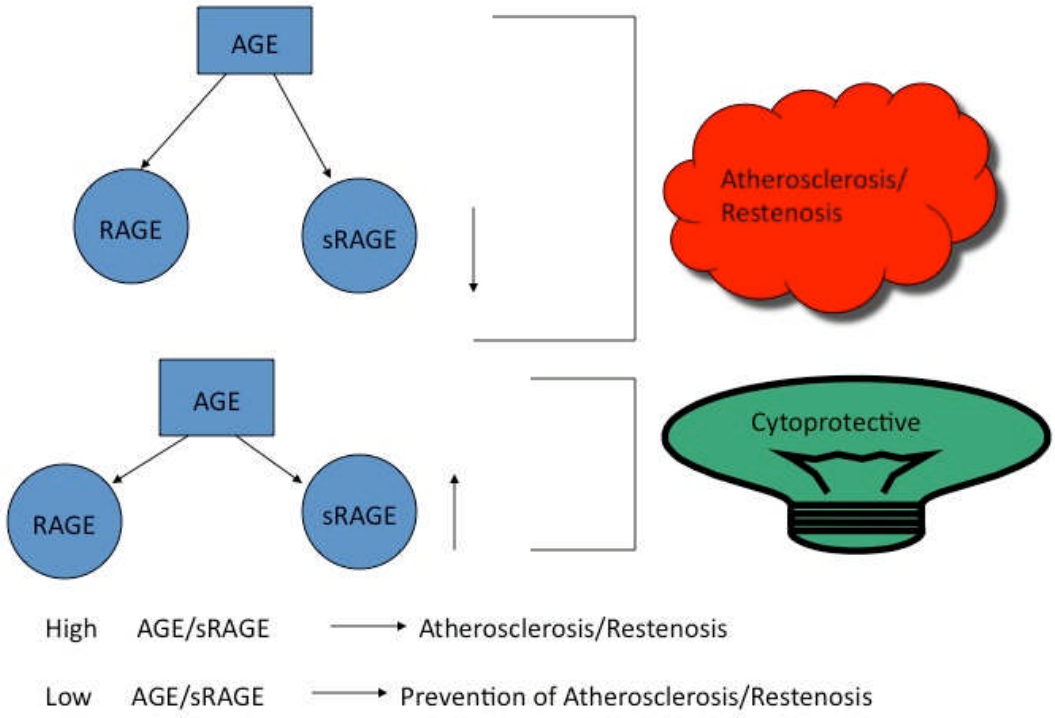


Figure 1. The mechanism by which the AGE/sRAGE ratio determines either stenosis or cytoprotection. AGE, advanced glycation end products, RAGE, receptor for advanced glycation end products, AGE/sRAGE, advanced glycation end products/soluble receptor for advanced glycation end products.

## 2.0 RATIONALE, HYPOTHESIS AND OBJECTIVES OF THE STUDY

### 2.1 Rationale

The use of stents has significantly improved the outcome of PCI; however, the long-term benefit of PCI is hampered by the possibility of restenosis of the treated segment (Nobuyoshi et al., 1988; Nobuyoshiet al., 1991; Hirshfeld et al., 1991; Waller, 1989). Intracoronary stent implantation along with balloon angioplasty is highly effective in the treatment of acute vessel closure (Sigwart et al., 1987; Roubin et al., 1992; de Jaegere et al., 1993a; Fischman et al., 1994). However, bare metal stents are thrombogenic, and the benefit achieved at the expense of high-risk vascular complications must be considered (Schatz et al., 1991; Serruys et al., 1994; de Jaegere et al., 1993b; Serruys et al., 1991). Despite major advances in PCI, in-stent restenosis remains a major limitation. Drug-eluting stents (DES) have emerged as a very promising approach in preventing restenosis and improving clinical outcome (Fischman et al., 1994; McKeage et al., 2003; Grube et al., 2003; Gershlick et al., 2004; Waugh and Wagstaff, 2004). However, the possibility of restenosis still exists. In-stent restenosis is now the most common form of restenosis. The incidence is between 25-45% with bare metal stents, and less than 10% with DES (Macaya et al., 1996; Stone et al., 2004). Up to half of restenosis (20-50%) is asymtomatic, but it may result in symptomatic myocardial ischemia. Although, stent-based therapeutics in clinical trials have shown markedly reduced rates of restenosis, the scope of clinically proven anti-restenotic agents is still extremely limited, and this requires additional strategies such as the administration of long-term antiplatelet medications (Morice et al., 2002; Teirstein and King, 2003; Eisenstein et al., 2007). The Late Angiographic Stent Thrombosis (LAST) study investigated the incidence of late angiographic stent thrombosis in an unselected DES population. The study concluded

that late stent thrombosis occured with an incidence of at least 35% in patients treated with DES despite long periods of antiplatelet therapy (McFadden et al., 2004; Andrew et al., 2004; Ong et al., 2005). Late angiographic stent thrombosis may also occur soon after dual antiplatelet therapy (clopidogrel and aspirin) is discontinued. However, there was no observation of LAST while patients were on long-term dual antiplatelet therapy (Pfisterer et al., 2006). Schofer et al. (2003) reported on the European multicenter, randomized, double blind study of the sirolimus-coated Cypher stent which was compared to the bare metal Bx velocity balloon expandable stent (E-SIRIUS) for the treatment of patients with de novo coronary lesions. The data after two years demonstrated that there was a significantly better clinical outcome concerning MACE for the Cypher group as compared to the bare metal group. However, later follow-up studies showed that one patient experienced an acute MI and cardiac rupture as a result of a localized hypersensitivity reaction, and late thrombosis of a sirolimus stent implanted 18 months earlier (Virmani et al., 2004). Following this incident and many others, the Food and Drug Administration, in association with Cordis Johnson & Johnson, posted adverse event information for physicians which stated that there had been more than 300 reports of subacute thrombosis (60 fatal) and fifty reports of hypersensitivity reaction with DES (US Food and Drug Administration Public Health Web Notification, 2003). This recent information led many cardiologists at the Royal University Hospital, Saskatoon, Saskatchewan, Canada to revert to bare metal stent implantation using DES for specific circumstances such as ostial and bifurcating lesions, diabetic patients and recurrent restenosis of bare metal stents.

Restenosis can be caused by numerous factors including arterial injury, inflammation, platelet-mediated thrombus formation, proliferation of SMC and vascular remodeling

(Waller, 1989). Smooth muscle cell proliferation, migration and collagen accumulation are major determinants of neointimal growth. Mediators of SMC proliferation are abundant, including ROS (Teirstein and King, 2003; Marumo et al., 1997; Sundaresen et al., 1995). SMC proliferation and stenosis after vascular injury coincide with elevations in ROS (Szocs et al., 2002; Shi et al., 2001). Reactive oxygen species have been detected in growth signaling pathways and in SMC proliferation (Colavitti et al., 2002; Abe and Berk, 1999; Bhunia et al., 1997). Reactive oxygen species have been implicated in the development of hypercholesterolemic atherosclerosis (Prasad and Kalra, 1993; Prasad, 1999; Prasad et al., 1994; Prasad, 2005; Prasad, 1997b; Prasad, 1998). In studies on cholesterol-fed rabbits, antioxidants attenuate/prevent the development of atherosclerosis. (Prasad and Kalra, 1993; Prasad, 1999; Prasad et al., 1994; Prasad, 2005; Prasad, 1997a). The sources of oxygen radical production during PCI could be numerous including xanthine-xanthine oxidase, mitochondria, polymorphonuclear leukocytes, arachidonic acid metabolites, homocysteine, C reactive protein and AGE-RAGE interaction (Prasad, 1999; Prasad, 1997a; McCord, 1985; Prasad, 2003a; Prasad, 1999; Misra, 1974; Prasad, 2004; Bierhaus et al., 2005). It is probable that ROS may be significantly involved in restenosis following PCI (McNair et al., 2006).

The results of Bose et al. (2007) demonstrated that patients who developed post-PCI restenosis with a decrease in lumen diameter of greater than 50% had significantly higher serum TNF-α levels after PCI as compared to those patients who developed post-PCI restenosis with a decrease in lumen diameters of less than 50%. In addition, their results showed that TNF-α is released into the aspirate of the stented SVG and that this release is correlated to the reduction of plaque volume and restenosis. TNF-α has been

suggested to be involved in the increase in RAGE expression in neointimal formation central to the development of restenosis (Takeda et al., 2005). Thus, it is plausible that increased levels of TNF-α may be involved in restenosis following PCI.

Numerous investigators have reported the contribution of sVCAM-1 to the development of post-PCI restenosis. Pigott et al. (1992) showed that the concentration of sVCAM-1 reflects the increased expression of VCAM-1 on the endothelial cell surface. The current knowledge outlining the expression of adhesion molecules by endothelial cells and SMCs during the development of restenosis was reported in studies on rabbit aortas. Balloon injury and resultant denudation of rabbit aortas induced ICAM-1 expression on endothelial cells two days post-trauma. In neointimal SMC the same procedure produced an increased expression of ICAM-1 and VCAM-1 at five to ten days which remained constant for at least 30 days after injury (Tanaka et al., 1993; Tanaka et al., 1995). During restenosis medial SMC experience a phenotypic change from a silent contractile to a synthetic cell type similar to fetal smooth muscle cells (Ardehali et al., 1995; Owens, 1995). These smooth muscle cells then proliferate and migrate from the media to the intima of adjacent arterial walls where they begin to divide and generate extracellular matrix, resulting in the production of intimal lesions (Peterson et al., 2007). Cytokine-stimulated SMC migration is regulated by the expression of VCAM-1, which interacts with the actin cytoskeleton and the extracellular matrix (Moiseeva, 2001). Currently, studies on the mouse injury model have determined that VLA-4 (counterreceptor for VCAM-1) is present on SMC and that VCAM-1 is necessary for the accumulation of SMC in the neointima (Barringhaus et al., 2004; Dansky et al., 2001). The inhibition of the VCAM-1/VLA-4 pathway attenuates monocyte adhesion,

infiltration and neointimal generation following arterial injury (Oguchi et al., 2000; Barringhaus et al., 2004). Peterson et al. (2007) suggested that the VLA-4/VCAM-1 interaction increases the proliferation of SMC in response to stimuli such as collagen and/or fibronectin (Nguyen, et al., 2005). Smooth muscle cell migration and neointimal proliferation are the hallmarks of post-PCI restenosis. Since sVCAM-1 reflects the increased expression of VCAM-1 on endothelial cells and VCAM-1 expression is essential for SMC migration and neointimal proliferation it follows that sVCAM-1 may play a role in post-PCI restenosis.

Experiments on wild type mice demonstrated that arterial endothelial denudation resulted in up-regulated RAGE in injured vessels and increased AGE accumulation in developing neointimal smooth cells. However, the administration of sRAGE lowered neointimal expansion, SMC proliferation and migration and release of MMPs (Sakaguchi et al., 2003). In addition, Zou et al. (2003) showed that the levels of AGEs and RAGE in the carotid arterial endothelium were higher in Zucker diabetic rats as compared to euglycemic controls. They induced injury to the carotid arteries of these rats by balloon inflation which increased the concentrations of AGEs and RAGEs, and resulted in neointimal hyperplasia. However, treatment with sRAGE before and up to 21 days post balloon damage significantly attenuated the neointimal development. This evidence suggests that sRAGE may be involved in the prevention of restenosis following PCI.

Restenosis is a major problem for long-term success after PCI such angioplasty and stenting. Restenosis following PCI is associated with neointimal hyperplasia. Balloon injury in the carotid artery and arterial endothelial denudation in animal models increase the levels of RAGE and AGEs in the arterial wall and produce neointimal hyperplasia.

The interaction of RAGE and AGEs results in the expression of adhesion molecules, cytokines, NF-κB and MMPs, and increased levels of tissue factor and oxidative stress. These substances are involved in the development of atherosclerosis, clot formation and plaque instability. sRAGE in animal models reduces neointimal growth and decreases smooth muscle cell proliferation and migration, and expression of extracellular matrix proteins. sRAGE reduced the atherosclerotic lesion in diabetic mice and this effect was associated with decreases in aortic VCAM-1, tissue factor, and MMP-9. sRAGE acts as a decoy for RAGE ligands and competes with full-length RAGE for ligand binding and thereby protects from the deleterious effects of the AGE and RAGE interaction.

The primary cause of acute coronary syndrome is thrombosis and the principal underlying pathology is atherosclerosis (often complicated by plaque rupture). Consistent with a protective anti-inflammatory effect of elevated levels of sRAGE is the observation that high levels of sRAGE are present in the sera of people who have extreme longevity. It is possible that levels of serum sRAGE are further lowered or are very low in patients with coronary artery disease who develop post-PCI restenosis as compared to those who do not develop post-PCI restenosis. Little is known about the levels of sRAGE in post-PCI restenosis. Since the combination of AGE, RAGE and sRAGE determines the extent of vascular injury, the measurement of these factors would be appropriate in determining the vascular complications. However, RAGE, a cell surface protein on the endothelium, cannot be measured. Measurements of sRAGE and AGE can be made. Not only the levels of sRAGE, but also the ratio of AGE/sRAGE might serve as a predictor of restenosis.

Since the interaction of RAGE with AGE results in an increased expression of cytokines and adhesion molecules, and since sRAGE attaches to AGEs, it is expected that low

sRAGE levels would be associated with an increase in levels of cytokine (TNF-α) and adhesion molecule (sVCAM-1).

Since stenosis is a common pathway for NSTEMI and post-PCI restenosis, this project has been divided into two parts: (A) serum sRAGE and NSTEMI; and (2) serum sRAGE and post-PCI restenosis.

## 2.2 HYPOTHESIS

### 2.2.1 Hypothesis for serum sRAGE and NSTEMI

It is hypothesized that:

(a) The levels of serum sRAGE are lower in patients with NSTEMI as compared to healthy controls.

(b) Low levels of serum sRAGE are associated with high levels of AGE, AGE/sRAGE, TNF-α and sVCAM-1.

(c) Serum sRAGE levels are inversely related to the number of affected vessels.

(d) Serum sRAGE levels are inversely related to the total plaque burden (volume).

(e) Low levels of serum sRAGE and high AGE/sRAGE ratio may serve as a predictor/biomarker for NSTEMI.

### 2.2.2 Hypothesis for serum sRAGE and post-PCI Restenosis

It is hypothesized that:

(a) Post-PCI restenosis is associated with low pre-PCI levels of serum sRAGE.

(b) Serum levels of sRAGE are negatively correlated with serum levels of AGE, AGE/RAGE ratio, TNF-α and sVCAM-1.

(c) Post-PCI serum levels of sRAGE will be lower than pre-PCI levels in patients who develop restenosis.

(d) Low pre-PCI serum levels of sRAGE and high AGE/sRAGE ratio may serve as a predictor/biomarker for post-PCI restenosis.

(e) Low post-PCI serum levels of sRAGE may serve as a predictor/biomarker for post-PCI restenosis.

## 2.3 OBJECTIVES

### 2.3.1 Objectives for serum sRAGE and NSTEMI

The objectives of this study were to investigate:

(1) If levels of serum sRAGE are lower and the levels of serum AGEs and the ratio of AGE/sRAGE are higher in NSTEMI patients compared to healthy subjects.

(2) If low levels of serum sRAGE and high levels of serum AGEs and AGEs/sRAGE ratio are associated with high levels of serum TNF-α and sVCAM-1.

(3) If the severity of coronary lesions (the number of affected vessels and plaque volume) is negatively correlated with serum sRAGE and positively correlated with serum TNF-α, sVCAM-1, AGE and AGE/sRAGE ratio.

(4) The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of sRAGE and AGE/sRAGE for the diagnosis of NSTEMI.

(5) If the low levels of sRAGE or high ratio of AGE/sRAGE is a better biomarker/predictor of NSTEMI.

### 2.3.2 Objectives for serum sRAGE and post-PCI Restenosis

The objectives of this study were to determine:

(1) Whether the pre-PCI serum levels of sRAGE are lower while those of sVCAM-1, TNF-α, AGE and AGE/sRAGE ratio are higher in NSTEMI subjects who develop post-PCI restenosis as compared to those who do not.

(2) Whether the post-PCI levels of sRAGE are lower than the pre-PCI levels in patients who develop post-PCI restenosis.

(3) Whether the post-PCI levels of TNF-α and sVCAM-1 are higher than those of pre-PCI levels in patients who develop post-PCI restenosis.

(4) Whether post-PCI levels of sRAGE, TNF-α, and sVCAM-1 are similar to pre-PCI levels in subjects who do not develop restenosis.

(5) The sensitivity, specificity, positive predictive value, negative predictive value and accuracy of sRAGE and AGE/sRAGE for the diagnosis of post-PCI restenosis.

(6) Whether the pre-PCI levels of sRAGE or AGE/sRAGE are better predictors/biomarkers for post-PCI restenosis.

To achieve the above objectives the studies were conducted in 46 consecutive NSTEMI patients and 28 control (healthy) subjects. Blood samples were collected for the measurement of AGE, sRAGE, TNF-α, and sVCAM-1 before PCI and 6-months post-PCI using enzyme linked immunosorbent assay (ELISA) kits. The details are given in the methods section.

## 3.0 PATIENTS AND METHODS

### 3.1 PATIENT SELECTION

Forty-six consecutive, Caucasian, male, patients with diagnosed NSTEMI ACS (by the attending Cardiologist) signed a consent form to participate in the study. The University of Saskatchewan Biomedical Research Ethics Board approved the study protocol (Bio #06-190). The Royal University Hospital Ethics Committee also approved the study. The clinical characteristics of the study patients were obtained through direct interviews with the patients prior to the procedure or with permission, from the patients' files. The demographic and clinical characteristics are summarized in Table 1. All patients of the study met the following inclusion criteria: (1) ACS patients who are of the NSTEMI subclass; (2) patients who have discrete denovo localized lesions in single vessel (1VD), double vessels (2VD) or triple vessels (3VD); (3) patients who received implantation of bare metal stents; (4) age range from 40-70 years; (5) non diabetic; and (6) male in gender. The exclusion criteria for patients in the study were as follows: (1) the reference diameter artery less than 2.5 mm in diameter; (2) acute MI within the previous five days; (3) post-coronary artery bypass graft surgery; (4) diabetic; (5) female in gender; (6) coexisting cardiomyopathy; (7) coexisting inflammatory diseases; (8) coexisting valvular disease (9) patients with a history of substance abuse; and (10) patients living more than 3hrs outside of the Saskatoon metropolitan area (patients living outside of the Saskatoon metropolitan area were less-likely to return for follow-up). The control group consisted of 28 age-matched healthy male hospital employees who had no history of the following (1) CAD; (2) HTN; (3) diabetes or (4) inflamatory diseases.

As mentioned earlier, the study is comprised of two parts: (1) serum sRAGE levels and NSTEMI; and (2) serum sRAGE levels and post-PCI restenosis. Forty-six consecutive

NSTEMI patients and 26 control subjects were enrolled in this study. All patients who underwent PCI were scheduled to return for a follow-up angiogram and blood work at 6-months or any time within 6-months if they developed recurrent symptoms (angina). The criterion for clinical coronary restenosis was angina with angiographic evidence of greater than 50% narrowing of the vessel (Hansrani et al., 2002; Kuntz and Baim, 1993; McNair et al., 2006).

## 3.2 METHODS

### 3.2.1 Methods for Angiographic Analysis

Coronary angiography is an invasive procedure using an iodine-based contrast medium which is injected under fluoroscopy to show the coronary anatomy and identify any areas of stenosis in the coronary arteries. Quantitative Coronary Analysis (QCA) is a computer-assisted angiographic method which affords an accurate objective evaluation of absolute and relative coronary artery dimensions during angiography. Angiography was performed and the angiograms analyzed by two observers blinded to the clinical characteristics of the patients. Reference diameters, minimal lumen diameter, percentage of stenosis and lesion length were measured using a semi-automated edge counter detection computer analysis system (Philips quantitative coronary angiography (QCA) DCI-ACA system, Netherlands). Coronary angiography showed that 16 patients had single vessel disease (1VD), 15 patients had double vessel disease (2VD), and 15 patients had triple vessel disease (3VD). The total lesion volume was calculated taking into consideration the length and diameter and the percent stenosis of the diseased segment of the vessel. For lesion volume calculation, the lesion was considered concentric (cylindrical) and the following modified formula was used: Lesion Volume = $\pi r^2 l$ x (1 - % stenosis), where $\pi$ = 3.14, r = radius of the diseased segment; l = length of the diseased segment, and % stenosis = percent of stenosis of the diseased segment. The total lesion volume for each patient was calculated by summation of their individual lesion volumes.

### 3.2.2 Methods for Determination of sRAGE

#### Principles of ELISA for sRAGE

The most common method for the measurement of sRAGE uses an enzyme immunoassay (EIA) that takes advantage of the catalytic properties of enzymes to detect and quantify immunological reactions. The enzyme-linked immunosorbent assay (ELISA), also called the sandwich enzyme immunoassay, is a type of EIA that is used to identify and assess the amount of antigen or antibody present in a sample (Kricka, 2001). To date there are two commercially available ELISA assays for the detection of human circulating sRAGE. The ELISA for total sRAGE uses antibodies that detect total circulating sRAGE. The other ELISA identifies esRAGE only using polyclonal antibodies raised against the distinct C-terminus of the esRAGE sequence (Sakurai et al., 2006). In the present study total circulating soluble RAGE was measured.

The principle of the sandwich ELISA is based upon the following: (1) the sample is added to the microtiter wells (a plate pre-coated by the manufacture with a known quantity of trapping antibody specific for the antigen in question) and any antigen present will bind to the antibody on the plate; (2) a detecting antibody is then added which binds to the antigen: (3) an enzyme-linked secondary antibody is added which binds to the detecting antibody; (4) a substrate is added which is then transformed by the enzyme into a fluorescent signal or color change which may be used as an indicator; (5) the measurement of the absorbance or fluorescence of the signal produced by the plate wells is proportional to the quantity of antigen present in the sample (Quantikine, Human sRAGE Immunoassay, R&D Systems, Minneapolis, MN USA; Kricka, 2001; Inoue et al., 1992).

3.2.2.1 Serum Preparation for sRAGE, AGE, TNF-α and sVCAM-1 ELISA

Blood was collected from NSTEMI patients pre-PCI and 6 months post-PCI (prior to follow-up angiogram). Seventeen mL of blood was collected from each patient or healthy control and transferred into 8.5 mL vacutainer serum separator tubes (EM Science, Merk KgA, Germany). Blood samples were allowed to clot and then immediately centrifuged at 1000 RPM for 15 minutes for serum collection. Serum was aliquoted into labeled Eppendorf tubes and stored at -80°C until assay was performed. The serum was used for the measurement of sRAGE, AGE, TNF-α and sVCAM-1.

Reagents for the sRAGE ELISA

*RAGE Microplate*

The 96-well polystyrene microplate consisted of 12 columns of 8 wells which were layered with a mouse monoclonal antibody raised (clones of a single parent cell) against RAGE (Rang, 2003).

*RAGE Conjugate*

The RAGE conjugate consisted of 21 ml of polyclonal antibody raised against RAGE and incorporated with horseradish peroxidase and preservatives.

*RAGE Standard*

The RAGE standard consisted of 50 ng of recombinant human RAGE/Fc Chimera. Chimeras are artificially produced by physically mixing cells from two different organisms. The RAGE standard consisted of a mixure of cells from the extracellular domain of mouse RAGE fused to the Fc region of human IgG1 via a peptide linker in a buffer with lyophilized preservatives. The RAGE standard was reconstituted with 1.0 mL of deionized water to yield a stock solution of 50,000 pg/mL (Strain et al., 1998).

*Serial Dilution of the RAGE Standard*

Seven new test tubes were labeled for sRAGE standard as follows: (1) 5,000 pg/mL; (2) 2,500 pg/mL; (3) 1,250 pg/mL; (4) 625 pg/mL; (5) 312 pg/mL; (6) 156 pg/mL; and (7) 78 pg/mL, respectively. 500 µL of the calibrator diluent was pipetted into each of the tubes labeled 2,500 pg/mL, 1,250 pg/mL, 625 pg/mL, 312 pg/mL, 156 pg/mL, and 78 pg/mL, respectively. Nine hundred µL of calibrator diluent and 100 µL of stock solution were then pipetted into the tube labeled 5,000 pg/mL. This solution was thoroughly mixed, and 500 µL was serially diluted down to the 78 pg/mL solution, respectively. The 5,000-pg/mL standard functioned as a high standard while the calibrator diluent served as the zero standard.

*Assay Diluent*

The assay diluent was composed of 11 mL of a buffered protein base with blue dye and preservatives.

*Calibrator Diluent*

The calibrator diluent was comprised of 21 mL of a buffered protein base with preservatives.

*Wash Buffer Solution*

The temperature of the concentrated wash buffer solution was allowed to reach room temperature to dissolve any crystals that may have formed in the solution.

Twenty mL of wash buffer concentrate was diluted with distilled water to a final volume of 500 mL.

*Substrate Solution*

The substrate solution contained an equal volume mixture (12.5 mL) of color reagents A and B which were prepared 15 minutes prior to use and protected from light exposure.

Color reagent A was made up of stabilized hydrogen peroxide, while color reagent B consisted of stabilized chromogen (tetramethylbenzidine).

*Stop Solution*

The solution to halt the reaction consisted of 6 mL of 2 N sulfuric acid (neutralizes the enzyme substrate reaction).

*Controls*

High (1,000 pg/mL, - 5,000 pg/mL) and low (50 pg/mL – 1000 pg/mL) lyophilized controls for human sRAGE were reconstituted with 1.3 mL of deionized water.

*Blanks Wells*

There were four blank wells that did not contain any samples, controls, or calibrators.

3.2.2.2 sRAGE Assay Procedure

The commercially available ELISA kit (Quantikine, Human sRAGE Immunosorbent assay, R&D Systems, Minneapolis, MN, USA) used for the determination of sRAGE consisted of a polystyrene microplate that was pre-coated with a monoclonal antibody specific for sRAGE. The 96-well microplate (12 x 8) was allowed to equilibrate at room temperature, while a template of the microplate was constructed on paper to act as a guide to identify the specific wells for blanks, calibrators, controls, and patient samples, all in duplicate. The ELISA was performed as follows:

- 100 µL of assay diluent were added to each well.
- 50 µL of serum, standards or controls were pipetted into the appropriate wells of the microplate and the immobilized antibody was bound to any existing sRAGE in the sample.
- The microplate was then covered with an adhesive strip and allowed to incubate for two hours at room temperature.

- Following incubation, each well of the microplate was aspirated and washed four times with 400 μL of wash buffer solution to eliminate any unbound substances.
- The plate was then inverted and blotted with Kleen Wipes.
- 200 μL of conjugate solution (detecting enzyme-linked polyclonal antibody specific for sRAGE) were added to each well and the microplate was covered with an adhesive strip and allowed to incubate for an additional two hours at room temperature.
- Following incubation, another aspiration/wash step was performed to displace any unbound antibody-enzyme reagent.
- With the addition of 200 μL of substrate solution to each well, a color evolved (from yellow to blue) in proportion to the amount of sRAGE bound in the initial step.
- Finally, the color development was terminated with 50 μL of stop solution. The color of the wells changed from blue to yellow following a gentle tapping along the walls of the microplate to ensure thorough mixing.
- The optical density with wavelength correction of each well was determined using a microplate reader set at 480 nm.

The microplate reader (Bio Tek ELx808) is an eight-channel, automated, bench-top, general purpose EIA analyzer with a curve-fitting statistical software program. The wavelength ranges between 380 to 900 nm with filters of 405, 450, 490, and 630 nm. The absorbance measurements range from 0.000 to 4.000 OD. The light source utilizes a tungsten-halogen-filled bulb. The microplate reader uses the same principle as a spectrophotometer; however it allows samples to be analyzed three times as fast. Therefore, labor, costs and disposables are significantly reduced (R & D Systems, 2005).

3.2.2.3 Calculation of Results

Blank, standard, control, and sample wells were performed in duplicate. The average duplicate readings of the blanks were subtracted from the average duplicate readings of the standards, controls and samples to derive the final optical density reading. A standard curve was constructed by summarizing the data using computer software (Gen 5$^{TM}$ Revision B, Biotek Instruments, Inc., Winooski, VT, USA) capable of producing a four-parameter logistic curve fit. Specifically, a standard curve was made by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, and formulating a best fit curve through the points on the graph. Various mathematical models have been created to derive standard curves for use within ELISAs. However, there is no general agreement on which curve-fitting method is the best one to use routinely with this immunoassay. The manufacturer of the ELISA kit suggested the four-parameter logistic log curve. Standard curves in both radioimmunoassay and ELISAs produce a sigmoidal shape when the optical density is plotted as a function of log dilution. The four parameter logistic function follows the same shape and is an accepted relationship to use in modeling standard curves. According to Plikaytis et al (1991) the four-parameter logistic log function provides the most dependable measure of antibody levels over the widest range of serum dilutions.

3.2.3 Methods for Determination of TNF-α

Principles of ELISA for TNF-α

The most common approach for the measurement of TNF-α uses a commercially available quantitative sandwich enzyme immunosorbentassay (ELISA) kit (Quantikine HS, Human TNF- α Immunoassay, R&D Systems, Minneapolis, MN, USA). The kit consists of a microplate that has been pre-coated with a monoclonal antibody specific for

TNF-α. Serum, standards and controls were pipetted into the wells of the microplate so that the immobilized antibody can bind any existing TNF-α. Following rinsing with a wash buffer solution, any unbound substances were eliminated. Next, an enzyme-linked polyclonal antibody specific for TNF-α was pipetted into the wells. Another wash step was then carried out to displace any unbound antibody-enzyme reagent. Subsequent to the addition of a substrate solution to the wells, and a one-hour incubation period, an amplifier solution was added to each well and a color evolved in proportion to the amount of TNF-α bound in the initial step. Finally, the color development was stopped with stop solution and the intensity of the color was immediately measured by a microplate reader set at 450 nm (R&D Systems, 2005).

Reagents for the TNF-α ELISA

*TNF-α Microplate*

The 96-well polystyrene microplate consisted of 12 columns of 8 wells that were layered with mouse monoclonal antibodies raised against TNF-α.

*TNF-α Conjugate*

The TNF-α conjugate consisted of 21 mL of polyclonal antibody raised against TNF-α in a solution of alkaline phosphatase and preservatives.

*TNF-α Standard*

The TNF-α standard consisted of 160 pg of recombinant human TNF-α in a buffered protein base with lyophilized preservatives. The TNF-α standard was reconstituted with 1.3 mL of calibrator diluent to produce a stock solution of 32 pg/mL.

*Serial Dilution of the TNF-α Standard*

Six polypropylene test tubes for TNF-α standard were labeled as follows: (1) 16 pg/mL; (2) 8 pg/mL; (3) 4 pg/mL; (4) 2 pg/mL; (5) 1 pg/mL; and (6) 0.5 pg/mL, respectively. 500 μL of the calibrator diluent was added to each tube. Then, 500 μL of the stock solution (32pg/mL) was added to the tube labelled 16 pg/mL. This solution was thoroughly mixed and 500 μL was serially diluted down to the 0.5 pg/ml standard. The 32 pg/mL undiluted standard functioned as a high standard while the calibrator diluent served as the zero standard (0 pg/mL).

*Assay Diluent*

The assay diluent was composed of 6 mL of a buffered protein base with preservatives.

*Calibrator Diluent*

The calibrator diluent was comprised of 21 mL of a buffered protein base with preservatives.

*Wash Buffer Solution*

The temperature of the concentrated wash buffer solution was allowed to reach room temperature to dissolve any crystals that may have formed in the solution.

One hundred mL of wash buffer concentrate was diluted with distilled water to make a final volume of 1000 mL.

*Substrate Solution*

The substrate solution was composed of lyophilized substrate (NADPH with stabilizers) and substrate diluent (buffered solution with stabilizers). The lyophilized substrate was reconstituted with 6 mL of substrate diluent.

*Amplifier Solution*

The amplifier solution was composed of an amplifier (lyophilized amplifier enzymes with stabilizers) and an amplifier diluent (buffered solution consisting of INT-violet with stabilizers). Reconstitution of the amplifier with 6.0 mL of amplifier diluent produced the amplifier solution.

*Stop Solution*

The solution to stop the reaction consisted of 6 mL of 2 N sulfuric acid.

*Controls*

High (55-100 pg/mL), medium (25-55 pg/mL) and low (1.5-25 pg/mL) lyophilized controls for human TNF-α were reconstituted with 1.3 mL of deionized water.

*Blanks Wells*

There were four blank wells that did not contain any samples, controls, or calibrators.

3.2.3.1 High Sensitive TNF-α Assay Procedure

The reagents, samples, and standards were prepared as in the previous section. The ELISA was performed as follows:

- 50 µL of assay diluent were added to each well.
- Next, 200 µL of standard, control or sample were added to each well.
- The microplate was then covered with an adhesive strip and allowed to incubate for three hours at room temperature.
- Following incubation, each well of the microplate was aspirated and washed six times with 400 µL of wash buffer solution.
- The plate was then inverted and blotted with Kleen Wipes.

- 200 μL of conjugate solution were added to each well and the microplate was covered with an adhesive strip and allowed to incubate for two hours at room temperature.

- After incubation, another aspiration/wash step was performed.

- Next, 50 μL of substrate solution were added to each well, and an adhesive strip was applied to the microplate which was then allowed to incubate for 1 hour at room temperature.

- 50 μL amplifier solution were added to each well and a color change (yellow to blue) developed. The plate was covered with an adhesive strip and allowed to incubate at room temperature for 30 minutes.

- Finally, 50 μL of stop solution were added to each well. The color of the wells remained the same.

- The optical density of each well was immediately determined using a microplate reader set at 490 nm.

3.2.3.2 Calculation of Results

Each blank, standard, control, and sample assay was performed in duplicate. The average duplicate readings of the blanks were subtracted from the average duplicate readings of the standards, controls and samples to derive the final optical density readings. A standard curve was constructed by summarizing the data using computer software (Gen $5^{TM}$ Revision B, Biotek Instruments, Inc. Winooski, Vermont, USA) capable of producing a four-parameter logistic curve fit. Specifically, a standard curve was made by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, and formulating a best fit curve through the points on the graph.

### 3.2.4 Methods for Determination of sVCAM-1

#### Principles of ELISA for sVCAM-1

The measurement of sVCAM-1 used a commercially available quantitative sandwich enzyme immunosorbent assay (ELISA) kit (Quantikine, Human sVCAM-1 Immunoassay, R&D Systems, Minneapolis, MN, USA). The kit consists of a microplate that has been pre-coated with a monoclonal antibody specific for sVCAM-1. Serum, standards and controls were pipetted into the wells of the microplate and the immobilized antibody binds any existing sVCAM-1. Following washing with a wash buffer solution, any unbound substances are eliminated. Next, an enzyme-linked polyclonal antibody specific for sVCAM-1 was pipetted into the wells. Another wash step was then carried out to displace any unbound antibody-enzyme reagent. Subsequent to the addition of a substrate solution to the wells, a color evolved in proportion to the amount of sVCAM-1 bound in the initial step. Finally, the color development was halted, and the intensity of the color was measured by a microplate reader set at 540 nm (R&D Systems, 2005).

#### Reagents for the sVCAM-1 ELISA

*sVCAM-1 Microplate*

The 96-well polystyrene microplate consisted of 12 columns of 8 wells which were layered with a mouse monoclonal antibody against sVCAM-1.

*sVCAM-1 Conjugate*

The sVCAM-1 conjugate consisted of 11 mL of polyclonal antibody raised against sVCAM-1 and incorporated with horseradish peroxidase and preservatives.

*sVCAM-1 Standard*

The sVCAM-1 standard consisted of 400 ng/vial of recombinant human sVCAM-1 in a buffer with lyophilized preservatives. The sVCAM-1 standard was reconstituted with 1 mL of deionized water to produce a stock solution of 400 ng/mL.

*Serial Dilution of the sVCAM-1 Standard*

Seven new test tubes for sVCAM-1 standard were labeled as follows: (1) 400 ng/mL; (2) 200 ng/mL; (3) 100 ng/mL; (4) 50 ng/mL; (5) 25 ng/mL; (6) 12.5 ng/mL; and (7) 6.25 pg/mL, respectively. 500 µL of the calibrator diluent was pipetted into each of the tubes. 500 µL of the sVCAM-1 stock solution was added to the tube labelled 400 ng/mL. This solution was thoroughly mixed and 500 µL serially diluted down to the 6.25 ng/mL standard. The 400 ng/mL stock solution functioned as a high standard while the calibrator diluent served as a zero standard (0 ng/mL).

*Calibrator Diluent*

The calibrator diluent consisted of 21 mL of a buffered protein base with preservatives.

*Wash Buffer Solution*

The temperature of the concentrated wash buffer solution was allowed to reach room temperature to dissolve any crystals that may have formed in the solution. Twenty mL of wash buffer concentrate was diluted using distilled water to make 500 mL of wash buffer solution.

*Substrate Solution*

The substrate solution (25 mL) contained an equal volume mixture (12.5 mL) of color reagents A and B which was prepared 15 minutes prior to use and protected from light exposure. Color reagent A was composed of stabilized hydrogen peroxide, while color reagent B consisted of stabilized chromogen (tetramethylbenzidine).

*Stop Solution*

The solution to stop the reaction consisted of 6 mL of 2 N sulfuric acid.

*Controls*

High (500-1000 ng/mL) and low (0-500 ng/mL) lyophilized controls for human sVCAM-1 were reconstituted with 1.3 mL of deionized water.

*Blanks Wells*

There were four blank wells that did not contain any samples, controls, or calibrators.

3.2.4.1 sVCAM-1 Assay Procedure

- The reagents, samples and standards were prepared as in the previous section.

- One hundred mL of sVCAM-1 conjugate were added to each well.

- Next, 100 μL of standards, controls or samples were added to each well. The microplate was then covered with an adhesive strip and allowed to incubate for one and a half hours at room temperature.

- Following incubation, each well of the microplate was aspirated and washed four times with 400 μL of wash buffer solution. The plate was then inverted and blotted with Kleen Wipes.

- 100 μL of substrate solution were added to each well and the microplate was covered with an adhesive strip and allowed to incubate for twenty minutes in the dark at room temperature. A color change from yellow to blue occurred after the addition of the substrate solution.

- 50 μL of stop solution were added to each well. The color of the wells changed from blue to yellow following a gentle tapping along the walls of the microplate to ensure thorough mixing. The optical density with wavelength correction of each well was determined using a microplate reader set at 540 nm.

3.2.4.2 Calculation of Results

The calculations of the concentration of sVCAM-1 in the standards, controls, and samples were made by subtracting the average zero standard optical density from the average of the duplicate readings. A standard curve was constructed by summarizing the data using computer software (Gen $5^{TM}$ Revision B, Biotek Instruments, Inc. Winooski, Vermont, USA) capable of producing a four-parameter logistic curve fit. The standard curve was made by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, and formulating a best fit curve through the points on the graph. The samples were diluted, and the concentrations read from the standard curve were multiplied by the dilution factor (20x).

3.2.5 Methods for Determination of AGE

Principles of ELISA for AGE

The principle of the sandwich ELISA is based upon the following: (1) the sample is added to the microtiter wells [a plate pre-coated by the manufacture with a known quantity of trapping antibody specific for the antigen in question] and any antigen present will bind to the antibody on the plate; (2) a detecting antibody is then added which binds to the antigen: (3) an enzyme-linked secondary antibody is added which binds to the detecting antibody; (4) a substrate is added which is then transformed by the enzyme into a fluorescent signal or color change which may be used as an indicator; (5) the measurement of the absorbance or fluorescence of the signal produced by the plate wells is proportional to the quantity of antigen present in the sample (BIOPCR, BeiJing Zhong Hao Shidai Co., Ltd, Repubic of China, 2009).

Reagents for the AGE ELISA

*AGE Microplate*

The 96-well polystyrene microplate consisted of 12 columns of 8 wells which were layered with a mouse monoclonal antibody raised (clones of a single parent cell) against AGE (Rang, 2003).

*AGE Conjugate*

The AGE conjugate consisted of 21 ml of polyclonal antibody raised against AGE and incorporated with horseradish peroxidase and preservatives.

*AGE Standard*

The AGE standard consisted of 50 ng of recombinant human AGE/Fc Chimera. Chimeras are artificially produced by physically mixing cells from two different organisms. The AGE standard consisted of a mixure of cells from the extracellular domain of mouse AGE fused to the Fc region of human IgG1 via a peptide linker in a buffer with lyophilized preservatives. The AGE standard was reconstituted with 1.0 mL of deionized water to yield a stock solution of 500 ng/mL (Strain et al., 1998).

*Serial Dilution of the AGE Standard*

Seven new test tubes were labeled for AGE standard as follows: (1) 500 ng/mL; (2) 250 ng/mL; (3) 125 ng/mL; (4) 62.5 ng/mL; (5) 31.2 ng/mL; (6) 15.6 ng/mL; and (7) 7.8 ng/mL, respectively. 500 μL of the calibrator diluent was pipetted into each of the tubes labeled 250 ng/mL, 62.5 ng/mL, 31.2 ng/mL, 15.6 ng/mL, and 7.8 ng/mL, respectively. 500 μL of stock solution was then pipetted into the tube labeled 500 ng/mL. This solution was thoroughly mixed, and 500 μL was serially diluted down to the 7.8 ng/mL solution respectively. The 500 ng/mL standard functioned as a high standard while the calibrator diluent served as the zero standard.

*Assay Diluent*

The assay diluent was composed of 11 mL of a buffered protein base with blue dye and preservatives.

*Calibrator Diluent*

The calibrator diluent was comprised of 21 mL of a buffered protein base with preservatives.

*Wash Buffer Solution*

The temperature of the concentrated wash buffer solution was allowed to reach room temperature to dissolve any crystals that may have formed in the solution.

Twenty mL of wash buffer concentrate was diluted with distilled water to a final volume of 500 mL.

*Substrate Solution*

The substrate solution contained an equal volume mixture (12.5 mL) of color reagents A and B which were prepared 15 minutes prior to use and protected from light exposure. Color reagent A was made up of stabilized hydrogen peroxide, while color reagent B consisted of stabilized chromogen (tetramethylbenzidine).

*Stop Solution*

The solution to halt the reaction consisted of 6 mL of 2 N sulfuric acid (neutralized the enzyme substrate reaction).

*Controls*

High (100 ng/mL, - 800 ng/mL) and low (5 ng/mL – 100 ng/mL) lyophilized controls for human AGE was reconstituted with 1.3 mL of deionized water.

*Blanks Wells*

There were four blank wells that did not contain any samples, controls, or calibrators.

#### 3.2.5.1 AGE Assay Procedure

The commercially available ELISA kit (BIOPCR, BeiJing ZhongHao Shidai Co., Ltd, Repubic of China, 2009) used for the determination of AGE consisted of a polystyrene microplate that was pre-coated with a monoclonal antibody specific for AGE. The 96-well microplate (12 x 8) was allowed to equilibrate at room temperature, while a template of the microplate was constructed on paper to act as a guide to identify the specific wells for blanks, calibrators, controls, and patient samples, all in duplicate. The ELISA was performed as follows:

- 100 μL of assay diluent were added to each well.
- 50 μL of serum, standards or controls were pipetted into the appropriate wells of the microplate and the immobilized antibody was bound to any existing AGE in the sample.
- The microplate was then covered with an adhesive strip and allowed to incubate for two hours at room temperature.
- Following incubation, each well of the microplate was aspirated and washed four times with 400 μL of wash buffer solution to eliminate any unbound substances.
- The plate was then inverted and blotted with Kleen Wipes.
- 200 μL of conjugate solution (detecting enzyme-linked polyclonal antibody specific for AGE) were added to each well and the microplate was covered with an adhesive strip and allowed to incubate for an additional two hours at room temperature.
- Following incubation, another aspiration/wash step was performed to displace any unbound antibody-enzyme reagent.

- With the addition of 200 μL of substrate solution to each well, a color evolved (from yellow to blue) in proportion to the amount of AGE bound in the initial step.
- Finally, the color development was terminated with 50 μL of stop solution. The color of the wells changed from blue to yellow following a gentle tapping along the walls of the microplate to ensure thorough mixing.
- The optical density with wavelength correction of each well was determined using a microplate reader set at 450 nm.

The microplate reader (Bio Tek ELx808) is an eight-channel, automated, bench-top, general purpose EIA analyzer with a curve-fitting statistical software program. The wavelength ranges between 380 to 900 nm with filters of 405, 450, 490, and 630 nm. The absorbance measurements range from 0.000 to 4.000 OD. The light source utilizes a tungsten-halogen-filled bulb. The microplate reader uses the same principle as a spectrophotometer; however it allows samples to be analyzed three times as fast. Therefore, labor, costs and disposables are significantly reduced (R & D Systems, 2005).

3.2.5.2 Calculation of Results

Blank, standard, control, and sample wells were performed in duplicate. The average duplicate readings of the blanks were subtracted from the average duplicate readings of the standards, controls and samples to derive the final optical density reading. A standard curve was constructed by summarizing the data using computer software (Gen 5$^{TM}$ Revision B, Biotek Instruments, Inc., Winooski, VT, USA) capable of producing a four-parameter logistic curve fit. Specifically, a standard curve was made by plotting the mean absorbance for each standard on the y-axis against the concentration on the x-axis, and formulating a best fit curve through the points on the graph. Various

mathematical models have been created to explain standard curves for use within ELISAs. However, there is no general agreement on which curve-fitting method is the best one use routinely with this immunoassay. The manufacturer of the ELISA kit suggested the four-parameter logistic log curve. Standard curves in both radioimmunoassay and ELISAs produce a sigmoidal shape when the optical density is plotted as a function of log dilution. The four parameter logistic function follows the same shape and is an accepted relationship to use in modeling standard curves. According to Plikaytis et al. (1991), the four-parameter logistic log function provides the most dependable measure of antibody levels over the widest range of serum dilutions. The method for analysis of the lipid profile (total cholesterol, HDL-cholesterol, LDL-cholesterol, triglycerides) was via a timed endpoint enzyme assay. The instrument used for the lipid profile was a Beckman-Coulter DXE 800. The method of analysis of hs-CRP was accomplished by near infrared immunoassay and it also used a Beckman-Coulter DXE 800 instrument. The method of analysis for serum glucose was the $O_2$-rate method which is based upon the theory that the peak rate of $O_2$ consumed is directly proportional to the concentration of glucose present. The instrument used to measure glucose was also the Beckman-Coulter DXE 800.

3.2.5 Methods for the Determination of the Diagnositic: Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value and Accuracy

The results obtained from a diagnostic test can be categorized into the following groups: (1) True positive (TP)- patients that get a positive test result and have the disease, (2) True Negative (TN)- individuals who get a negative test result and do not have the disease; (3) False Positive (FP)- individuals who get a positive test result and do not have the dsease; and (4) False Negative (FN)- patients who get a negative test result and

have the disease. The sensitivity is the probability that a test result will be positive in patients with the disease [(TP/TP + FN) x 100] whereas, the specificity is the probability that a test result will be negative in individuals without the disease (TN/TN + FP) x 100. The positive predictive value (PPV) is the probability that a patient will have a disease given a positive test result [(TP/TP + FP) x 100] whereas, the negative predictive value (NPV) is the probability that an individual will not have a disease given a negative test result (TN/ FN + TN) x 100. Finally, the accuracy is the probability of correctly identified subjects [ TP + TN/ TP + TN + FP+FN x 100] (Glas et al., 2003).

### 3.3 STATISTICAL ANALYSIS

Statistical analyses were performed utilizing the Statistical Program for the Social Sciences (SPSS) version 13.0. Sample size was calculated using a two-sided Satterthwaite t-test to have 95% power and alpha level of 5%. The results were expressed as mean ± standard error of the mean (SEM). In addition, the unpaired t-test was used to compare data between groups. Single linear univariate correlations (Spearman's Correlation Coefficients) were performed to evaluate the relationships between circulating sRAGE levels and the following variables: serum TNF-α, sVCAM-1, AGE, AGE/sRAGE and extent of coronary lesion. The serum levels of sRAGE, TNF-α, sVCAM-1, AGE, AGE/sRAGE in 1VD, 2VD and 3VD were compared with control. The serum levels of sRAGE, AGE, AGE/sRAGE, TNF-α and sVCAM-1 of the restenosis groups were compared with those of the non-restenosis group. A value of $p < 0.05$ was considered significant.

## 4.0 RESULTS

## A. Serum sRAGE and NSTEMI

### 4.1 Part I- Demographic and Clinical Characteristics of Control and NSTEMI Patients

4.1.1 Demographics of Control and NSTEMI Patients

*Blood Pressure*

Demographic and clinical characteristics of the control subjects and patients with NSTEMI are shown in Table 1. The systolic, diastolic and mean blood pressures were 125 ± 4.3, 78 ± 2.7, and 90 ± 13 mmHg, respectively in control subjects. The systolic, diastolic and mean blood pressures for NSTEMI patients were 150 ± 15.8, 68 ± 10.4 and 88 ± 12.72 mmHg, respectively. There was a significant difference ($p<0.001$) between the systolic and diastolic blood pressures of contols versus NSTEMI patients. Thirty-five of the 46 NSTEMI (76%) had a history of hypertension; however, none of the control subjects were hypertensive. All of the NSTEMI patients had a history of smoking.

*Body Mass Index (BMI)*

The body mass index (BMI) in control subjects and NSTEMI patients ranged from 18 to 32 $kg/m^2$ (mean 25 ± 1.5 $kg/m^2$) and 19 to 46 $kg/m^2$ (mean 29 ± 0.70 $kg/m^2$) respectively, and the values were not significantly different from each other.

4.1.3 High-Sensitivity C-Reactive Protein (hs-CRP)

The hs-CRP test is more sensitive than the standard CRP test because it can detect lower concentrations of the protein in the blood. The serum hs-CRP for control subjects and NSTEMI patients ranged from 1.0 to 5.0 (mean 3.0 ± 0.26) and 0.2 to 43.8 (mean 10.39 ± 2.17) mg/L respectively, and were significantly different ($p<0.001$) from each other.

*Serum Glucose*

The fasting serum glucose levels in control subjects and NSTEMI patients were 4.8 ± 0.32 and 5.4 ± 0.21 mmol/L respectively, and the values were not significantly different from each other.

Serum *Lipids*

Serum lipid profiles [total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), and cholesterol risk ratio (TC/HDL-C)] were measured in both control subjects and NSTEMI patients. Twenty-nine of 46 NSTEMI patients (63%) had a history of hypercholesterolemia. None of the control subjects had a history of hypercholesterolemia.

*Total Cholesterol (TC)*

Serum levels of total cholesterol in control subjects ranged from 4.3 to 5.20 (mean ± SE, 4.4 ± 0.25) mmol/L, while the levels in NSTEMI patients ranged from 4.30 to 8.53 (mean ± SE, 6.04 ± 0.25) mmol/L. The levels were significantly ($p < 0.05$) higher in NSTEMI patients compared to control subjects.

*HDL-C*

Serum levels of HDL-C in control subjects ranged from 0.95 to 1.74 (mean ± SE, 1.35 ± 0.14) mmol/L, while the levels in NSTEMI patients ranged from 0.43 to 2.7 (mean ± SE, 0.92 ± 0.30) mmol/L. The values were significantly ($p < 0.005$) higher in control subjects as compared to NSTEMI patients.

*LDL-C*

Serum levels of LDL-C in control subjects ranged from 2.51 to 3.30 (mean ± SE, 2.78 ± 0.10) mmol/L, while the levels of NSTEMI patients ranged from 1.7 to 4.38 (mean ±

SE, 4.33 ± 0.33) mmol/L. The values were significantly (p = 0.014) higher in NSTEMI patients compared to control subjects.

*Triglycerides (TG)*

Serum levels of triglycerides in control subjects ranged from 0.62 to 2.27 (mean ± SE, 2.19 ± 0.30) mmol/L, while the levels in NSTEMI patients ranged from 0.65 to 6.8 (mean ± SE, 2.6 ± 0.32) mmol/L. The values of the two groups were not significantly different from each other.

*Cholesterol Risk Ratio [Total cholesterol (TC)/ HDL-C]*

The TC/HDL-C ratio in control subjects in ranged from 3.6 to 4.78 (mean ± SE, 3.89 ± 0.33), while the ratio in NSTEMI patients ranged from 2.26 to 15.89 (mean ± SE, 7.62 ± 0.55). The values were significantly ($p < 0.001$) higher in NSTEMI patients compared to control subjects.

*Age*

The age of control subjects ranged from 42 to 69 (mean ± SE, 60 ± 2.0) years. The age of NSTEMI patients ranged from 40 to 70 (mean ± SE, 63.8 ± 1.5) years. There was no significant difference in age between the two groups.

The demographic data demonstrate that a significant number of subjects who developed NSTEMI ACS were hypertensive, hypercholesterolemic and had elevated CRP levels compared to control subjects.

**Table 1: Demographic and clinical characteristics of Controls and NSTEMI Patients.**

| Parameter | Controls<br>Range<br>Mean ± SEM | NSTEMI Patients<br>Range<br>Mean ± SEM | p Value |
|---|---|---|---|
| *Total Cholesterol (mmol/L)* | (4.30 - 5.20)<br>4.4 ± 0.25* | (4.30 - 8.53)<br>6.04 ± 0.25 | <0.05 |
| *High-Density Lipoprotein Cholesterol (mmol/L)* | (0.95 – 1.74)<br>1.35 ± 0.14* | (0.43 – 2.70)<br>0.92 ± 0.30 | <0.005 |
| *Low-Density Lipoprotein Cholesterol (mmol/L)* | (2.51 - 3.3)<br>2.78 ± 0.10* | (1.7- 4.38)<br>4.33 ± 0.33 | <0.014 |
| *Triglyceride (mmol/L)* | (0.62 – 2.27)<br>2.19 ± 0.30 | (0.65 – 6.8)<br>2.6 ± 0.32 | NS |
| *Cholesterol Risk Ratio* | (3.6 – 4.78)<br>3.89 ± 0.33* | (2.26 - 15.89)<br>7.62 ± 0.55 | <0.001 |
| *Fasting Glucose (mmol/L)* | (3.6 – 5.9)<br>4.8 ± 0.32 | (3.5 – 5.9)<br>5.4 ± 0.21 | NS |
| *Hs CRP (mg/L)* | (1.0- 5.0)<br>3.05 ± 0.26* | (0.2 - 43.8)<br>10.39 ± 2.17 | <0.001 |
| *Body Mass Index (kg/m$^2$)* | (18 – 32)<br>25 ± 1.5 | (19 – 46)<br>29 ± 0.70 | NS |
| *Blood Pressure (mmHg)* | Systolic= (110-140)<br>Mean Sys = 125<br>Diastol. Range=(70-85)<br>Mean Dia = 78<br>Mean Range = 85-95<br>Mean = 90 | Systolic= (100-180)<br>Mean Sys=150<br>Diastolic = (55-110)<br>Mean Dia=68<br>Mean Range=80-105<br>Mean = 88 | <0.001 |
| *Age (years)* | (42-69)<br>60 ± 2.0 | (40-70)<br>63.8± 1.5 | NS |

The results are expressed as range and mean ± SE

* p< 0.05, control vs. NSTEMI.

The ranges are shown in the brackets. Highly Sensitive C-reactive protein (Hs CRP), Non ST-elevation myocardial infarction, (NSTEMI).

### 4.1.2 Serum levels of sRAGE, sVCAM-1, TNF-α, AGE, and AGE/sRAGE in Control and NSTEMI Patients

*sRAGE*

The levels of serum sRAGE in control subjects and NSTEMI patients are summarized in Figure 1. The serum sRAGE levels of control subjects and NSTEMI patients were 1287 ± 41.5 and 884.55 ± 50 pg/mL, respectively. The values in NSTEMI patients were significantly ($p < 0.001$) lower than those in control subjects.

*sVCAM-1*

The serum levels of sVCAM-1 in control subjects and NSTEMI patients are summarized in Figure 1. The serum sVCAM-1 levels in control subjects were 651 ± 35.5 ng/mL, while the levels of NSTEMI patients were 1059.62 ± 70.8 ng/mL. The values in NSTEMI patients were significantly ($p < 0.0003$) higher as compared to control subjects.

*TNF-α*

The serum levels of TNF-α in control subjects and NSTEMI patients are summarized in Figure 1. The TNF-α levels in control subjects and NSTEMI patients were 10.3 ± 0.8 and 23.1 ± 2.3 pg/mL, respectively. The values in NSTEMI patients were significantly higher ($p < 0.002$) than those in control subjects.

*AGE*

The serum levels of AGE in control subjects and NSTEMI patients are summarized in Figure 1. The AGE levels of control subjects and NSTEMI patients were 669.40 ± 47.9 and 1192.50 ± 82.6 ng/mL respectively. The values in NSTEMI patients were significantly ($p < 0.001$) higher than those in control subjects.

*AGE/sRAGE Ratio*

The AGE/sRAGE ratios in control subjects and NSTEMI patients are summarized in Figure 1. The AGE/sRAGE ratio in control subjects was 0.52 ± 0.06, while the ratio in NSTEMI patients was 1.75 ± 0.17. The ratio was significantly ($p < 0.001$) higher in NSTEMI patients compared to control subjects.

In summary, these data indicate that the serum levels of sRAGE are lower, and those of TNF-α, sVCAM-1, AGE, and AGE/sRAGE are higher in NSTEMI patients compared to control subjects.

**sRAGE, TNF-α, sVCAM-1, AGE, and AGE/sRAGE in Control and NSTEMI Patients**

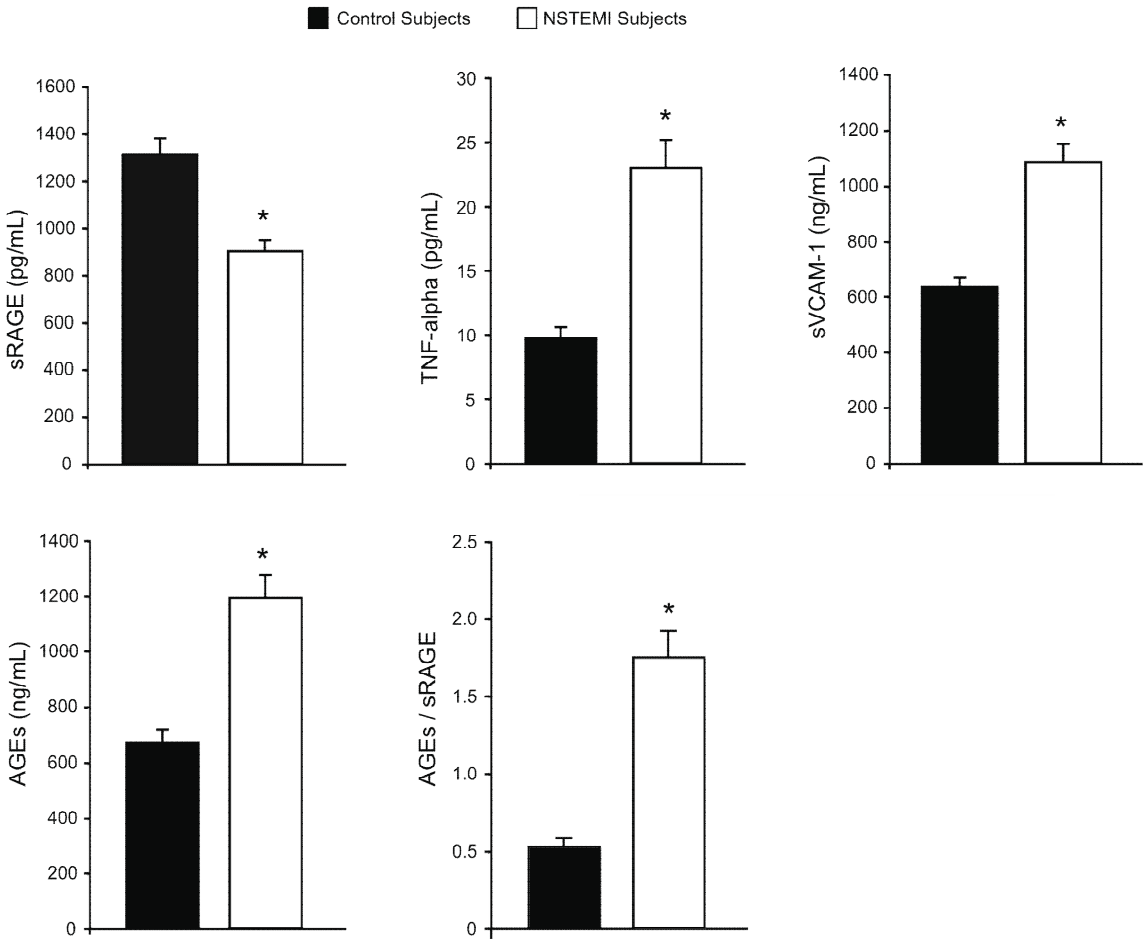


Figure 1. The results are expressed as mean ± SE. Serum levels of soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), soluble vascular cell adhesion molecule-1 (sVCAM-1), advanced glycation end products (AGEs) and advanced glycation end products/soluble receptor for advanced glycation end products (AGE/sRAGE) in control subjects and non-ST-elevation myocardial infarction (NSTEMI). * $p<0.05$, control vs. NSTEMI

### 4.1.3 Correlation of Coronary Lesion Volume with sRAGE, sVCAM-1, TNF-α, and AGE in NSTEMI Subjects

*sRAGE vs. Lesion Volume*

The serum sRAGE levels plotted against the volume of the lesions in NSTEMI patients are depicted in Figure 2. The Spearman correlation coefficient was - 0.79 with $p < 0.001$. There was an inverse relationship between lesion volume and serum sRAGE levels.

*sVCAM-1 vs. Lesion Volume*

The serum sVCAM-1 levels plotted against the volume of the lesions in NSTEMI patients are shown in Figure 2. The Spearman correlation coefficient was 0.53 with $p < 0.001$. Levels of sVCAM-1 were positively correlated with lesion volume.

*TNF-α vs. Lesion Volume*

The serum TNF-α levels plotted against the volume of the lesions in NSTEMI patients are shown in Figure 2. The Spearman correlation coefficient was 0.742 with $p < 0.001$. Serum levels of TNF-α were positively correlated with lesion volume.

*AGE vs. Lesion Volume*

The serum AGE values plotted against the volume of the lesions in NSTEMI patients are given in Figure 2. The Spearman correlation coefficient was 0.70 with $p < 0.001$. Serum levels of AGE were positively correlated with lesion volume.

In summary, these data indicate that the serum levels of sRAGE are negatively, while those of AGE, TNF-α, and sVCAM-1 are positively correlated with the lesion volume in NSTEMI patients.

**Correlation of Lesions with Various Biomarkers in NSTEMI Patients**

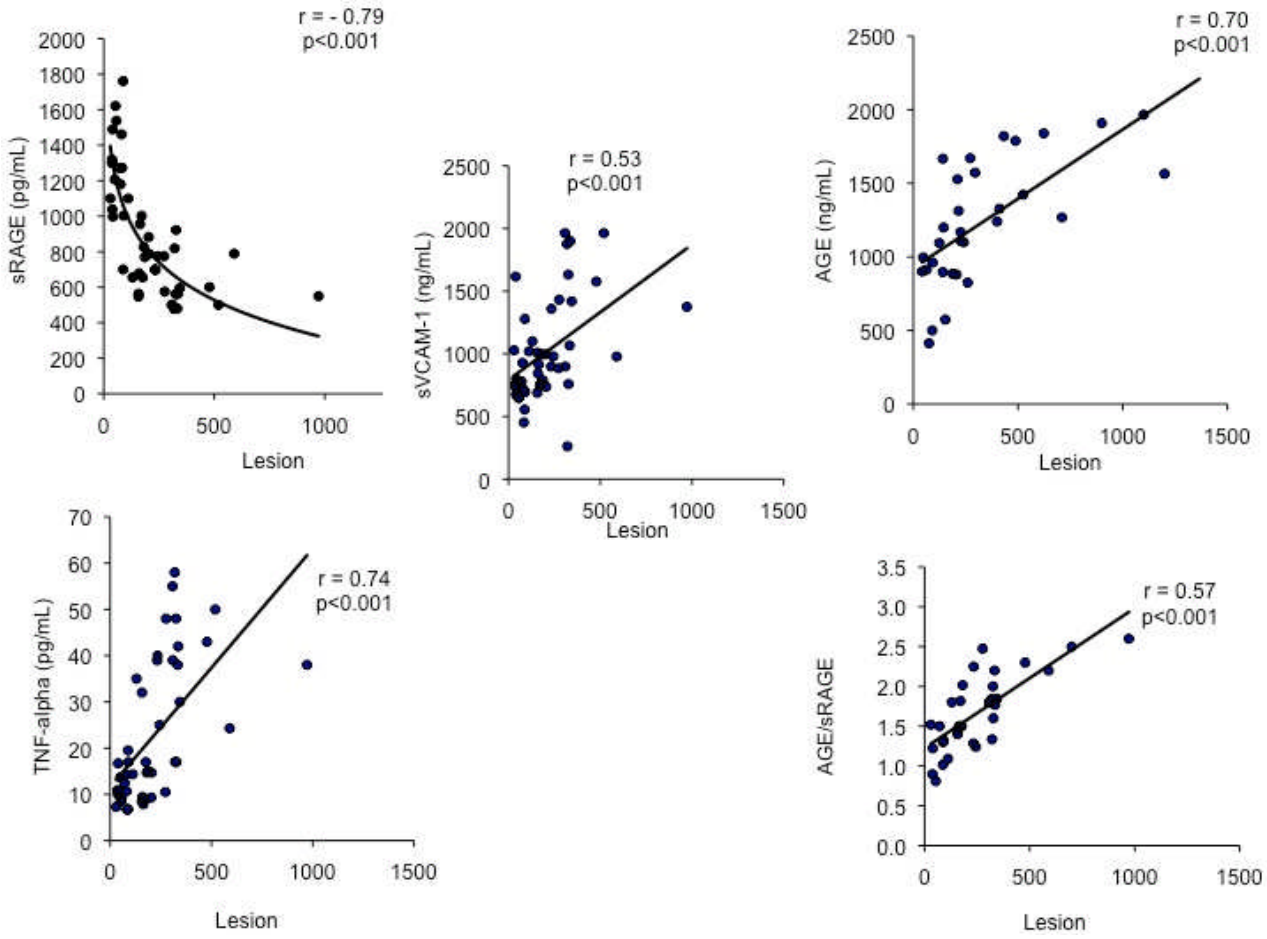


Figure 2. Correlation of coronary lesion volume with sRAGE, sVCAM-1, TNF-α, AGE and AGE/sRAGE in NSTEMI Subjects. Soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), soluble vascular cell adhesion molecule-1 (sVCAM-1), advanced glycation end products (AGEs) and advanced glycation end products/soluble receptor for advanced glycation end products (AGE/sRAGE). Lesion volume units are expressed as $mm^3$.

### 4.1.4 Relation of sRAGE, sVCAM-1, TNF-α, AGE and AGE/sRAGE with the Number of Affected Vessels in NSTEMI Patients

**NSTEMI Patients**

*sRAGE*

The levels of serum sRAGE in control and NSTEMI subjects with number of affected vessels are summarized in Figure 3. The serum sRAGE values of control subjects and NSTEMI patients with 1VD, 2VD, and 3VD were 1287.1 ± 41.5, 1020.8 ± 82.7, 956.0 ± 93.4 and 681.3 ± 51.8 pg/mL, respectively. The values were significantly higher in control subjects ($p < 0.001$) compared to NSTEMI patients with 1VD, 2VD, and 3VD. The values were not significantly different between patients with 1VD and 2VD.

The values were lower in patients with 3VD compared to those in patients with 1VD and 2VD.

*TNF-α*

The serum levels of TNF-α in control subjects and NSTEMI patients with number of affected vessels are summarized in Figure 3. The serum TNF-α values of the control subjects and NSTEMI patients with 1VD, 2VD, and 3VD were 10.3 ± 0.8, 15.3 ± 2.9, 19.1 ± 3.4, and 34.6 ± 3.4 pg/mL, respectively. The values of the the NSTEMI patients with 1VD, 2VD, and 3VD were significantly higher ($p < 0.001$) compared to the values of control subjects. The values were significantly higher ($p < 0.001$) in NSTEMI patients with 3VD compared to those with 1VD. The values were not significantly different between patients with 2VD and 1VD.

*sVCAM-1*

The serum levels of sVCAM-1 in control subjects and NSTEMI patients with number of affected vessels are summarized in Figure 3. The serum sVCAM-1 values of control

subjects and NSTEMI subjects with 1VD, 2VD, and 3VD were 651.0 ± 35.5, 843.06 ± 52.8, 1019.27 ± 90.8, and 1393.0 ± 138.4 ng/mL, respectively. The values of the NSTEMI patients with 1VD, 2VD, and 3VD were significantly higher ($p < 0.001$) compared to the values of the control subjects. The values were significantly higher ($p < 0.001$) in NSTEMI patients with 3VD compared to those with 1VD. The values were not significantly different between patients with 2VD and 1VD.

*AGE*

The serum levels of AGE in control subjects and NSTEMI patients with number of affected vessels are summarized in Figure 3. The serum AGE values of the control subjects and NSTEMI patients with 1VD, 2VD, and 3VD were 669.4 ± 47.9, 1201.8 ± 159, 1015.1 ± 119.3, and 1394.5 ± 145.3 ng/mL, respectively. The values in the NSTEMI patients with 1VD, 2VD, and 3VD were significantly higher ($p < 0.05$) compared to the values in the control subjects. However, there was no significant difference in the serum levels of AGE among patients with 1VD, 2VD, and 3VD.

*AGE/sRAGE Ratio*

The AGE/sRAGE ratios in control subjects and NSTEMI patients with number of diseased vessels are summarized in Figure 3. The ratio of the AGE/sRAGE of the control subjects and NSTEMI patients with 1VD, 2VD, and 3VD were 0.52 ±, 1.62 ± 0.33, 1.45 ± 0.23, and 2.2 ± 0.31. The values were significantly higher ($p<0.001$) in NSTEMI patients with 1VD, 2VD, and 3VD compared to control subjects.

In summary, these data indicate that the serum levels of sRAGE are negatively, while those of AGE, AGE/sRAGE, TNF-α, and sVCAM-1 are positively correlated with the number of affected vessels in NSTEMI patients.

**sRAGE, TNF-α, sVCAM-1, AGE, and AGE/sRAGE in Controls and Patients with Diseased Vessels**

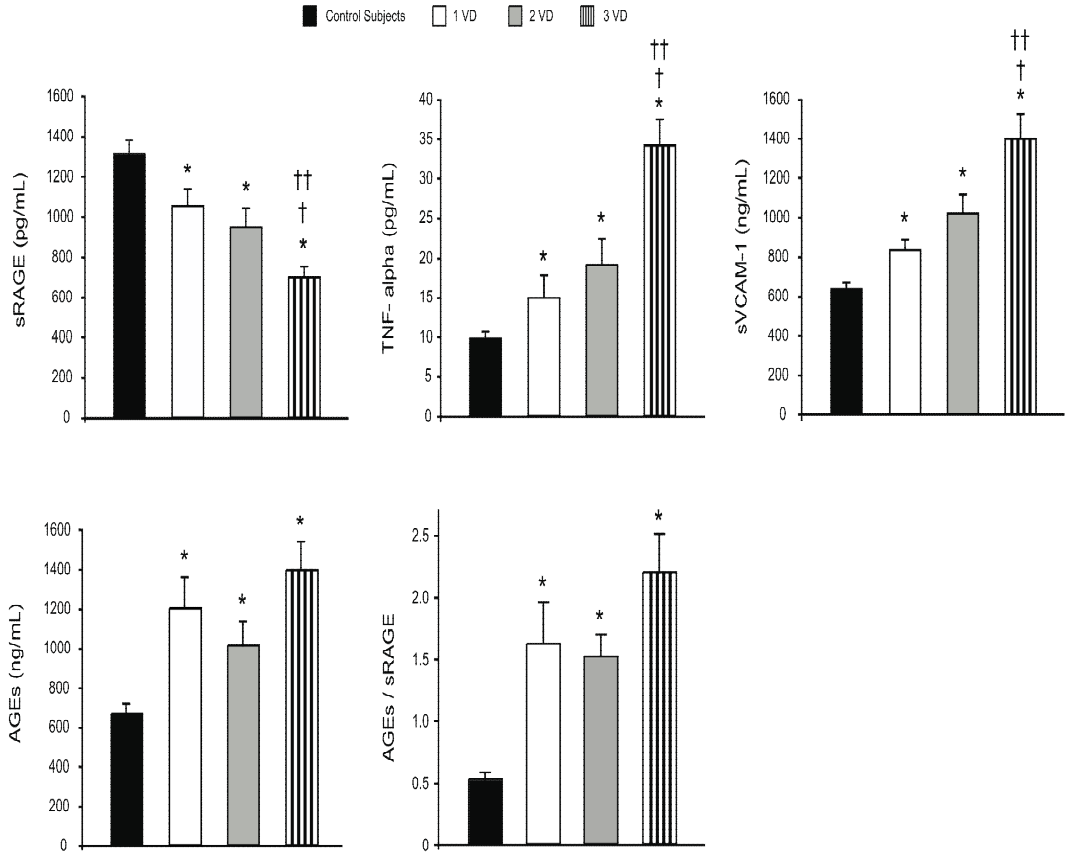


Figure 3. The serum levels of sRAGE, TNF-α, sVCAM-1, AGEs, and AGE/sRAGE in control subjects and NSTEMI patients with 1VD, 2VD, and 3VD. The results are expressed as mean ± SE. Soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), soluble vascular cell adhesion molecule-1 (sVCAM-1), advanced glycation end products (AGEs) and advanced glycation end products/soluble receptor for advanced glycation end products (AGE/sRAGE). 1VD, one vessel disease, 2VD, two vessel disease, 3VD, three vessel disease. ∗ $p < 0.05$, control vs. 1VD, 2VD or 3VD, † $p < 0.05$, 1 VDvs. 2VD or 3VD

†† $p<0.05$, 2VD vs. 3VD.

### 4.1.5 Correlation of Serum sRAGE with sVCAM-1, TNF-α, AGE and AGE/sRAGE in NSTEMI Subjects

*sRAGE vs. sVCAM-1*

The serum sRAGE levels plotted against the serum sVCAM-1 levels in NSTEMI patients are summarized in Figure 4. The Spearman correlation coefficient was -0.73 with $p< 0.001$. There was an inverse relationionship between sRAGE and sVCAM-1.

*sRAGE vs. TNF-α*

The serum sRAGE levels plotted against the serum TNF-α levels in NSTEMI patients are summarized in Figure 4. The Spearman correlation coefficient was -0.77 with $p< 0.001$. There was an inverse relationionship between sRAGE and TNF-α.

*sRAGE vs. AGE*

The serum sRAGE levels plotted against the serum AGE levels in NSTEMI patients are summarized in Figure 4. The Spearman correlation coefficient was -0.63 with $p< 0.001$. There was an inverse relationionship between sRAGE and AGE.

*AGE/sRAGE Ratio vs. sRAGE*

The AGE/sRAGE ratio plotted against the serum sRAGE levels in NSTEMI patients are summarized in Figure 4. The Spearman correlation coefficient was -0.79 with $p< 0.0001$. There was an inverse relationionship between sRAGE and AGE.

In summary, these data indicate that the serum levels of sRAGE are lower, while the levels of serum AGE, AGE/sRAGE, TNF-α, and sVCAM-1 are higher in patients with NSTEMI. The results also demonstrate that there is an inverse relationship between sRAGE and AGE, sRAGE and AGE/sRAGE, sRAGE and TNF-α, and sRAGE and sVCAM-1.

**Correlation between sRAGE and Various Biomarkers in NSTEMI Patients**

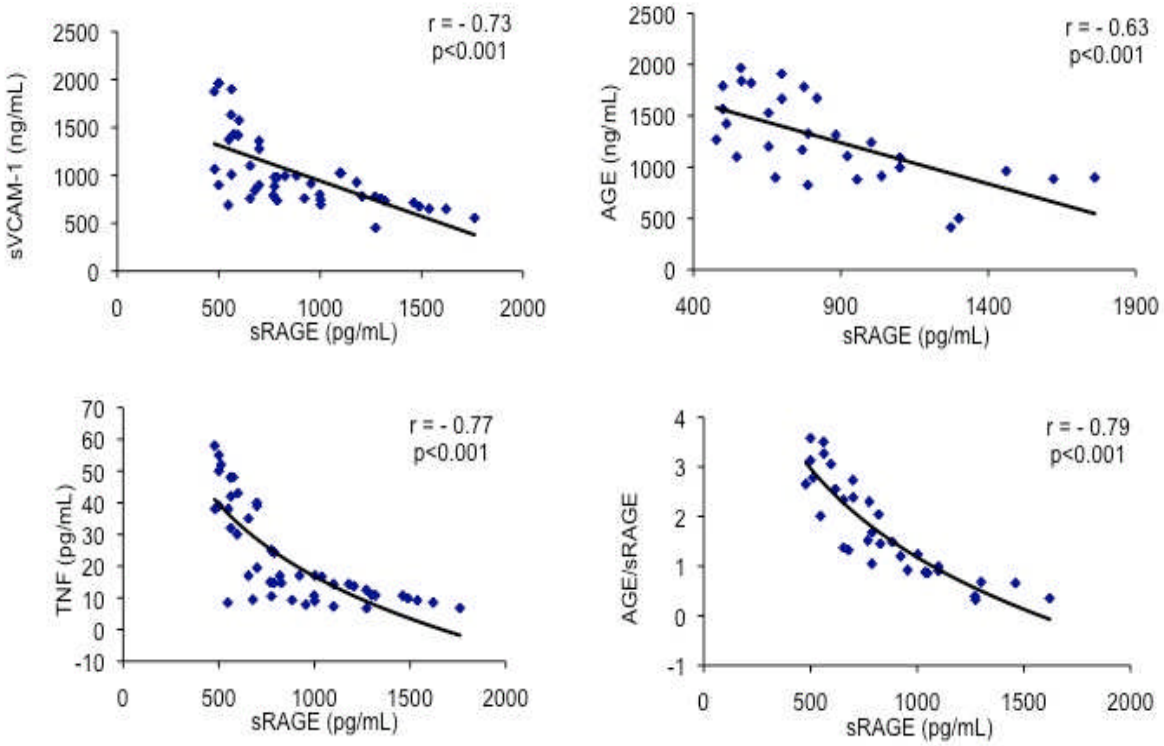


Figure 4. Correlation of sRAGE with sVCAM-1, TNF-α, AGE and AGE/sRAGE in NSTEMI Subjects. Soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), soluble vascular cell adhesion molecule-1 (sVCAM-1), advanced glycation end products (AGEs).

### 4.1.6 Sensitivity, Specificity, Predictive Values and Accuracy of the sRAGE and AGE/sRAGE Test

The sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy of the sRAGE biomarker test are summarized in Table 2. The mean – 2SD (848.7pg/mL) for sRAGE and the mean + 2SD (0.92) for AGE/sRAGE which covers 95% of the control subjects were taken as the cutoff points. The sensitivity, specificity, PPV, NPV, and accuracy of the sRAGE biomarker test were 59%, 100%, 100%, 100% and 74% respectively, while those of the AGE/sRAGE test were 85%, 91%, 97%, 67% and 86%, respectively, in the diagnosis of patients with NSTEMI acute coronary syndrome.

In summary, these data demonstrate that both sRAGE and AGE/sRAGE tests may serve as biomarkers/predictors for identifying patients with NSTEMI. However, the sensitivity of AGE/sRAGE appears to be greater than that of sRAGE, while the predictive value of the sRAGE is greater than that of the AGE/sRAGE.

**Table 2: Sensitivity, Specificity, Predictive Values and Accuracy of sRAGE and AGE/sRAGE tests for diagnosis of NSTEMI Patients.**

| Biomarker | Sensitivity | Specificity | PPV | NPV | Accuracy |
|---|---|---|---|---|---|
| **sRAGE** | **59%** | **100%** | **100%** | **100%** | **74%** |
| **AGE/sRAGE** | **85%** | **91%** | **97%** | **67%** | **86%** |

**S**ensitivity: The probability that a test result will be positive in patients with the disease.

Specificity: The probability that a test result will be negative in individuals without the disease.

Positive Predictive Value (PPV): The probability that a patient will have a disease given a positive test result.

Negative Predictive Value (NPV): The probability that an individual will not have a disease given a negative test result.

Accuracy: The probability of correctly identified subjects.

Non-ST-elevation myocardial infarction (NSTEMI), soluble receptor for advanced glycation end products (sRAGE), advanced glycation end products (AGE), receptor for advanced glycation end products (RAGE).

**Results**

**B. Serum sRAGE and post-PCI Restenosis**

## 4.2 PART II. Demographic and Clinical Characteristics of Patients with or without Restenosis

Out of 46 NSTEMI patients who underwent the PCI procedure 19 developed post-PCI restenosis and 27 did not develop restenosis.

4.2.1 Demographics of patients with or without Restenosis

Demographic and clinical characteristics of NSTEMI patients with or without restenosis are shown in Table 3.

*Blood Pressure*

The systolic, diastolic and mean blood pressures of were 148 ± 4.3, 68 ± 2.7, and 90 ± 13 mmHg, respectively in patients with restenosis. The systolic, diastolic and mean blood pressures for patients without restenosis were 153 ± 15.8, 70 ± 10.4 and 91 ± 12.72 mmHg, respectively. There was no significant difference between the two groups.

*Body Mass Index (BMI)*

The BMI values of patients who developed and who did not develop restenosis were 30.1 ± 1.0 and 27.4 ± 0.7 $kg/m^2$, respectively. The values in the patients with restenosis were significantly ($p < 0.04$) higher than in those without restenosis.

*Serum hs CRP*

The levels of serum hs CRP in the patients who developed restenosis were 12.3 ± 3.4 mg/L, while the levels in patients who did not develop restenosis were 8.4 ± 3.6 mg/L. The levels were significantly ($p < 0.02$) higher in patients with restenosis compared to the patients without restenosis.

*Serum Glucose*

The levels of fasting serum glucose of control subjects and NSTEMI patients were 5.4 ± 0.22 and 5.5 ± 0.25 mmol/L, respectively, and these values were not significantly different from each other.

*Total Cholesterol*

The levels of serum total cholesterol of patients who developed restenosis ranged from 4.08 to 8.53 (mean ± SE, 6.0 ± 0.30) mmol/L, while the levels of patients who did not develop restenosis ranged from 3.24 to 8.19 (mean ± SE, 4.5 ± 0.20) mmol/L. The levels were significantly ($p < 0.003$) higher in patients with restenosis compared to the patients without restenosis.

*HDL-Cholesterol*

The levels of serum HDL-C in patients who developed restenosis ranged from 0.43 to 1.8 (mean ± SE, 0.9 ± 0.10) mmol/L, while the levels in patients who did not develop restenosis ranged from 0.43 to 2.7 (mean ± SE, 1.0 ± 0.10) mmol/L. These values of the two groups were not significantly different from each other.

*LDL-Cholesterol*

The serum LDL-C levels in patients who developed restenosis ranged from 1.71 to 4.38 (mean ± SE, 4.30 ± 0.40) mmol/L, while the levels in patients who did not develop restenosis ranged from 1.7 to 4.30 (mean ± SE, 3.8 ± 0.30) mmol/L. The values of the two groups were not significantly different from each other.

*Triglyceride*

The levels of serum triglycerides in patients who developed restenosis ranged from 1.34 to 4.18 (mean ± SE, 2.5 ± 0.30) mmol/L, while the levels in patients who did not

develop restenosis ranged from 1.5 to 6.8 (mean ± SE, 2.4 ± 0.4) mmol/L. The values of the two groups were not significantly different from each other.

*Cholesterol Risk Ratio (TC/HDL-C)*

The TC/HDL-C ratio in patients with restenosis ranged from 3.5 to 5.0 (mean ± SE, 3.92 ± 0.37) while the ratio in patients without restenosis ranged from 3.6 to 4.9 (mean ± SE, 4.65 ± 0.52). The values were not significantly different between the two groups.

*Age*

The ages of patients who developed restenosis ranged from 40 to 70 (mean ± SE, 61.5 ± 2.9) years. The ages of patients who did not develop restenosis ranged from 49 to 70 (mean ± SE, 66.1 ± 1.9) years. There was no significant difference in age between the two groups.

These data indicate that the patients that developed restenosis were hypercholesterolemic and had significantly higher levels of serum CRP than those patients without restenosis.

**Table 3: Demographic and Clinical Characteristics of Patients with and without Restenosis**

| Parameter | With Restenosis N=19 (Range) Mean ± SEM | WithoutRestenosis N=27 (Range) Mean ± SEM | p Value |
|---|---|---|---|
| *Total Cholesterol (mmol/L)* | (4.08 – 8.53) 6.0 ± 0.30* | (3.24 - 8.19) 4.5 ± 0.20 | <0.003 |
| *HDL-C (mmol/L)* | (0.43 – 1.80) 0.9 ± 0.10 | (0.43 – 2.70) 1.0 ± 0.10 | NS |
| *LDL-C (mmol/L)* | (1.71 – 4.38) 4.38 ± 0.40 | (1.7- 4.30) 3.8 ± 0.33 | NS |
| *Triglyceride (mmol/L)* | (1.34 – 4.18) 2.5 ± 0.30 | (1.5 – 6.8) 2.4 ± 0.4 | NS |
| *Cholesterol Risk Ratio* | (3.5-5.0) 3.92 ± 0.37 | (3.6-4.9) 4.65 ± 0.52 | NS |
| *Fasting Glucose (mmol/L)* | (3.5 – 5.8) 5.4 ± 0.22 | (3.6 – 5.9) 5.5 ± 0.25 | NS |
| *Hs CRP (mg/L)* | (0.7- 42.5) 12.3 ± 3.4* | (0.2 - 43.3) 8.4 ± 3.6 | <0.01 |
| *Body Mass Index (kg/m$^2$)* | (18 – 32) 25 ± 1.5 | (19 – 46) 29 ± 0.70 | NS |
| *Blood Pressure (mmHg)* | Systolic = (110-175) Mean Sys = 148 Diastolic = (70-85) Mean Dia = 74 Mean Range = 85-95 Mean = 90 | Systolic = (100-180) Mean Sys = 153 Diastolic = (70-85) Mean Dia = 70 Mean Range = 85-97 Mean = 91 | NS |
| *Age (years)* | (40-70) 61.5 ± 2.9 | (49-70) 66.1 ± 1.9 | NS |

The results are expressed as range and mean ± SE, * p< 0.05, control vs. NSTEMI.

The ranges are shown in the brackets. Highly Sensitive C-reactive protein (HS CRP), Non ST-elevation myocardial infarction, (NSTEMI). High density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C).

### 4.2.2 Quantitative Coronary Angiography (QCA) Results

The pre-PCI, post-PCI and six-month follow-up values for QCA in-stent lesion characteristics for patients with and without restenosis are summarized in Table 4. The reference vessel diameter (RVD) is the diameter of the vessel adjacent to the segment of stenosis. The pre-PCI minimum lumen diameter (MLD) is defined as the diameter of the lumen before the intervention. The post-PCI minimum lumen diameter is the diameter of the lumen after the intervention. The acute gain (AG) is the increase in lumen diameter from the intervention. The percent diameter stenosis (%DS) is the degree of the stenotic lesion expressed as a percentage. Finally, the late lumen loss (LL) is the post-PCI MLD minus the follow-up MLD (Reiber et al., 1985; Biondi-Zoccai et al., 2008).

*Pre-PCI QCA Results*

The pre-PCI RVD values for patients with and without restenosis were 2.96 ± 0.16 and 3.0 ± 0.16 mm, respectively and these values were not significantly different from each other. The MLD values for patients with and without restenosis were 1.56 ± 0.06 and 1.49 ± 0.07 mm, respectively and these values were not significantly different from each other. The values for the percent diameter stenosis (%DS) for patients with and without restenosis were 76.74 ± 2.9 and 75.23 ± 3.9 %, respectively and these values were not significantly different from each other. The values for the lesion length of patients with and without restenosis were 19.38 ± 2.0 and 19.40 ± 1.9 mm, respectively. These values were not significantly diferent from each other.

*Post-PCI QCA Results*

The MLD values for patients with and without restenosis immediately following PCI were 2.93 ± 0.11 and 2.91 ± 0.12 mm, respectively and these values were not

significantly different from each other. The values for the percent diameter stenosis of patients with and without restenosis were 11.14 ± 0.34 and 10.85 ± 0.32 %, respectively and these values were not significantly different from each other.

*Six- Month Follow-up QCA Results*

The MLD values for patients with and without restenosis at the six-month follow-up were 2.20 ± 0.07 and 2.61 ± 0.10 mm, respectively and these values were significantly different ($p<0.004$) from each other. The values for the percent diameter stenosis of patients with and without restenosis were 55.1 ± 0.60 and 10.13 ± 0.38 %, respectively and these values were significantly different ($p< 0.001$) from each other. The values for late lumen loss (post-PCI of patients with and without restenosis were 0.73 ± 0.44 and 0.30 ± 0.48 mm, respectively. These values were significantly different ($p<0.001$) from each other. The values for the loss index of patients with and without restenosis were 0.53 ± 0.33 and 0.21 ± 0.27, respectively and these values were significantly ($p<0.003$) different from each other.

In summary, these data demonstrate that at six month follow-up the MLD was smaller and the percent diameter stenosis and late lumen loss was greater in the patients that developed restenosis as compared to those without restenosis.

**Table 4: Quantitative Coronary Angiography Results (QCA)**

| QCA In-Stent Lesion Characteristics | With Restenosis | Without Restenosis | p |
|---|---|---|---|
| ***Pre-PCI*** | | | |
| RVD (mm) | 2.96 ± 0.16 | 3.0 ± 0.16 | 0.648 |
| MLD (mm) | 1.56 ± 0.06 | 1.49 ± 0.07 | 0.354 |
| %DS | 76.74. ± 2.9 | 75.23±3.88 | 0.944 |
| Lesion Length (mm) | 19.38 ±1.96 | 19.40±1.88 | 0.960 |
| ***Post-PCI*** | | | |
| MLD (mm) | 2.93 ± 0.11 | 2.91 ± 0.12 | 0.91 |
| %DS | 11.14 ± 0.34 | 10.85 ± 0.32 | 0.54 |
| ***6-Month Follow-up*** | | | |
| MLD (mm) | 2.20 ± 0.07 | 2.61 ±0.10 | 0.004 |
| % DS | 55.2±0.60 | 10.13±0.38 | 0.001 |
| Late Lumen Loss (mm) | 0.73±0.44 | 0.30±0.48 | <0.001 |
| Loss Index | 0.53±0.33 | 0.21±0.27 | 0.003 |

The results are expressed as range and mean ± SE, * $p < 0.05$, with restenosis vs. without restenosis, reference vessel diameter (RVD), minimum lumen diameter (MLD), percent diameter stenosis (%DS).

### 4.2.3 Pre-PCI Levels of Serum sRAGE, TNF-α, sVCAM-1, AGE, and AGE/sRAGE in Patients with or without Restenosis

*sRAGE*

The pre-PCI levels of serum sRAGE in patients with and without restenosis are summarized in Figure 5. The pre-PCI serum sRAGE levels in patients with and without restenosis were 610.6 ± 24.1 and 1143.8 ± 52.5 pg/mL, respectively. These values were significantly different ($p < 0.001$) from each other.

*TNF-α*

The pre-PCI levels of serum TNF-α in patients with and without restenosis are summarized in Figure 5. The pre-PCI levels of serum TNF-α in patients with and without restenosis were 37.9 ± 2.5 and 11.6 ± 0.41 pg/mL, respectively. These values were significantly different ($p < 0.001$) from each other.

*sVCAM-1*

The pre-PCI levels of serum sVCAM-1 in patients with and without restenosis are summarized in Figure 6. The pre-PCI levels of serum sVCAM-1 in patients with and without restenosis were 1381.8 ± 63.5 and 811.37 ± 26.5 ng/mL, respectively. These values were significantly different ($p < 0.001$) from each other.

*AGE*

The pre-PCI levels of serum AGE in patients with and without restenosis are summarized in Figure 6. The pre-PCI levels of serum AGE in patients with and without restenosis were 1512.1 ± 84.53 and 891.7 ± 92.4 ng/mL, respectively. These values were significantly different ($p < 0.001$) from each other.

*AGE/sRAGE Ratio*

The pre-PCI levels of AGE/sRAGE in patients with and without restenosis are summarized in Figure 5. The pre-PCI levels of AGEs/RAGE in patients with and without restenosis were 2.39 ± 0.20 and 1.03 ± 0.17, respectively. These values were significantly different ($p < 0.001$) from each other.

In summary, these data show that the patients with restenosis had lower levels of serum sRAGE and higher levels of AGE, AGE/sRAGE, TNF-α, and sVCAM-1 compared to patients without restenosis.

**Pre-PCI Levels of Serum sRAGE, AGE, AGE/sRAGE, TNF-α, and sVCAM-1 in Patients with or without Restenosis**

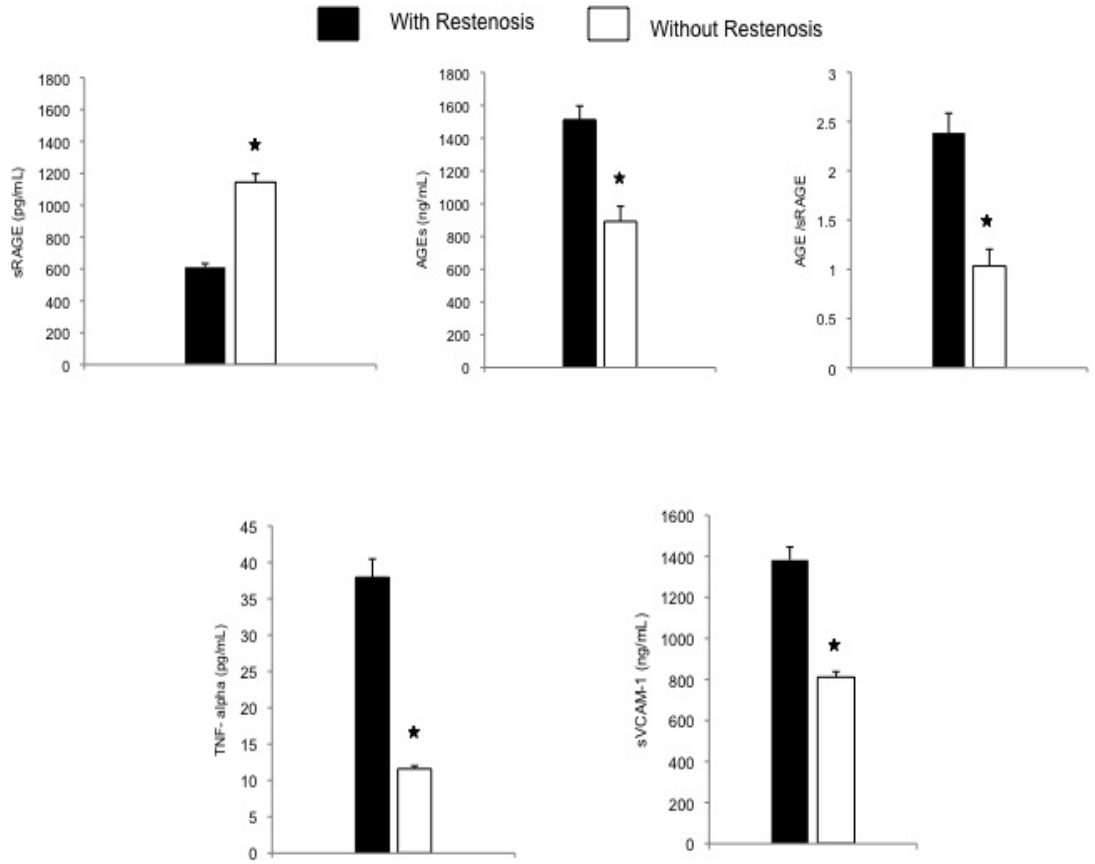


Figure 5. The pre-PCI levels of serum sRAGE, AGE, AGE/sRAGE, TNF-α, and sVCAM-1 in NSTEMI patients with or without restenosis. Soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), soluble vascular cell adhesion molecule-1 (sVCAM-1), advanced glycation end products (AGEs) and advanced glycation end products/soluble receptor for advanced glycation end products (AGE/sRAGE). Results are expressed as mean ± SE.

* $p < 0.05$, With Restenosis vs. Without Restenosis

### 4.2.4 Post-PCI Levels of Serum sRAGE, TNF-α and sVCAM-1 in Patients with or without Restenosis

*sRAGE*

The post-PCI levels of serum sRAGE in patients with and without restenosis are summarized in Table 5. The post-PCI levels of serum sRAGE in patients with and without restenosis were 477 ± 18.6 and 1106.7 ± 41.9 pg/mL, respectively. These values were significantly different ($p<0.0001$) from each other.

*TNF-α*

The post-PCI levels of serum TNF-α in patients with and without restenosis are summarized in Table 5. The post-PCI levels of serum TNF-α in patients with and without restenosis were 48.4 ± 1.4 and 12.5 ± 0.44 pg/mL, respectively. These values were significantly different ($p<0.0001$) from each other.

*sVCAM-1*

The post-PCI levels of serum sVCAM-1 in patients with and without restenosis are summarized in Table 5. The post-PCI levels of serum sVCAM-1 in patients with and without restenosis were 1381.8 ± 63.5 and 762.2 ± 26.4 ng/mL, respectively. These values were significantly different ($p<0.0001$) from each other.

In summary these data show that the post-PCI levels of serum sRAGE are lower, and those of sVCAM-1, and TNF-α are higher in patients with restenosis compared to those without restenosis.

**Table 5: Post-PCI Levels of Serum sRAGE, TNF-alpha, and sVCAM-1 in Patients with and without Restenosis.**

| Biomarker | With Restenosis | Without Restenosis | p Value |
|---|---|---|---|
| *sRAGE (pg/mL)* | 477 ± 18.6 | 1106.7 ± 41.9 | p<0.0001 |
| *TNF-alpha (pg/mL)* | 48.4 ± 1.4 | 12.5 ± 0.44 | p<0.0001 |
| *sVCAM-1 (ng/mL)* | 1381.8 ± 63.5 | 767.2 ± 26.4 | p<0.0001 |

The results are expressed as mean ± SE

Soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), soluble vascular cell adhesion molecule-1 (sVCAM-1).

4.2.5 Pre-and Post-PCI Levels of Serum sRAGE, TNF-α and sVCAM-1 in NSTEMI Patients with Restenosis.

*sRAGE*

The pre-and post–PCI levels of serum sRAGE in patients with restenosis are summarized in Figure 6. The pre-PCI and post-PCI levels of serum sRAGE in patients with restenosis were 610.6 ± 24.1 and 477 ± 18.6 pg/mL, respectively. These values were significantly different ($p < 0.001$) from each other.

*TNF-α*

The pre-and post–PCI levels of serum TNF-α in patients with restenosis are summarized in Figure 6. The pre-PCI and post-PCI levels of serum TNF-α in patients with restenosis were 37.9 ± 2.5, 48.4 ± 1.4 pg/mL, respectively and the values were significantly different ($p < 0.001$) from each other.

*sVCAM-1*

The pre-and post–PCI levels of serum sVCAM-1 in patients with restenosis are summarized in Figure 6. The pre-PCI and the post-PCI levels of serum sVCAM-1 in patients with restenosis were 1290 ± 99.3 and 1437.6 ± 155.0 ng/mL, respectively and the values were not significantly different from each other.

In summary, these results indicate that the post-PCI levels of serum sRAGE are lower, TNF- α levels are higher, and levels of sVCAM-1 are unchanged compared to pre-PCI levels in patients who developed restenosis.

**Pre-and Post-PCI Levels of Serum sRAGE, TNF-α and sVCAM-1 in Patients with Restenosis**

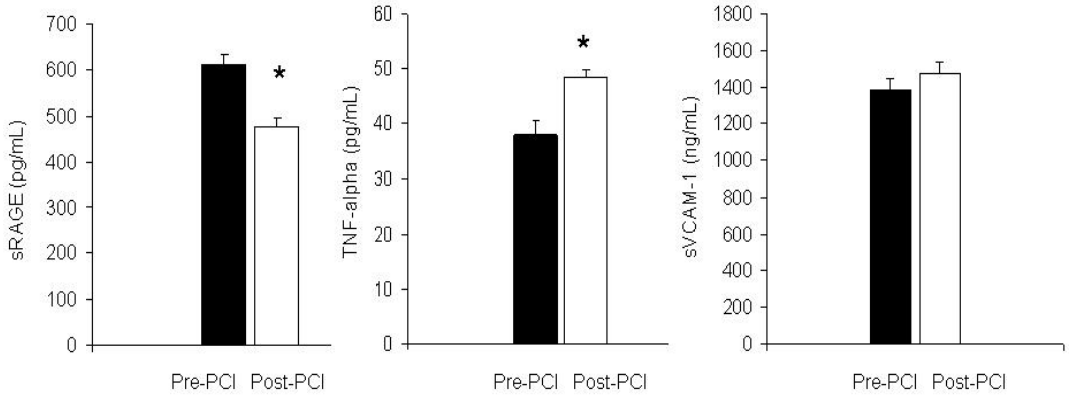


Figure 6. Pre-and Post-PCI Levels of Serum sRAGE, TNF-α and sVCAM-1 in NSTEMI Patients with Restenosis. Soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), soluble vascular cell adhesion molecule-1 (sVCAM-1). Pre- and post-procedural percutaneous coronary intervention (Pre-and Post-PCI). Results are expressed as mean ± SE.

* $p < 0.05$, Pre-PCI vs. Post-PCI.

4.2.6 Pre- and Post-PCI Serum Levels of sRAGE, TNF-α, and sVCAM-1 in NSTEMI Patients without Restenosis.

*sRAGE*

The pre- and post–PCI serum levels of sRAGE in patients without restenosis are summarized in Figure 7. The pre-PCI and post-PCI serum sRAGE levels in patients without restenosis were 1143.8 ± 52.5 and 1106.8 ± 41.9 pg/mL, respectively. These values were not significantly different from each other.

*TNF-α*

The pre- and post–PCI serum levels of TNF-α in patients without restenosis are summarized in Figure 7. The pre-PCI and post-PCI serum TNF-α levels in patients without restenosis were 11.6 ± 0.41, 12.5 ± 0.44 pg/mL, respectively and the values were not significantly different from each other.

*sVCAM-1*

The pre- and post–PCI serum levels of sVCAM-1 in patients without restenosis are summarized in Figure 7. The pre-PCI and the post-PCI serum sVCAM-1 levels in patients without restenosis were 811.4 ± 26.5 and 767 ± 26.4 ng/mL, respectively and the values were not significantly different from each other.

In summary, these results demonstrate that there is no significant change in the levels of sRAGE, TNF-α, and sVCAM-1 in NSTEMI patients without restenosis.

**Pre- and Post-PCI Serum Levels of sRAGE, TNF-α, and sVCAM-1 in Patients without Restenosis.**

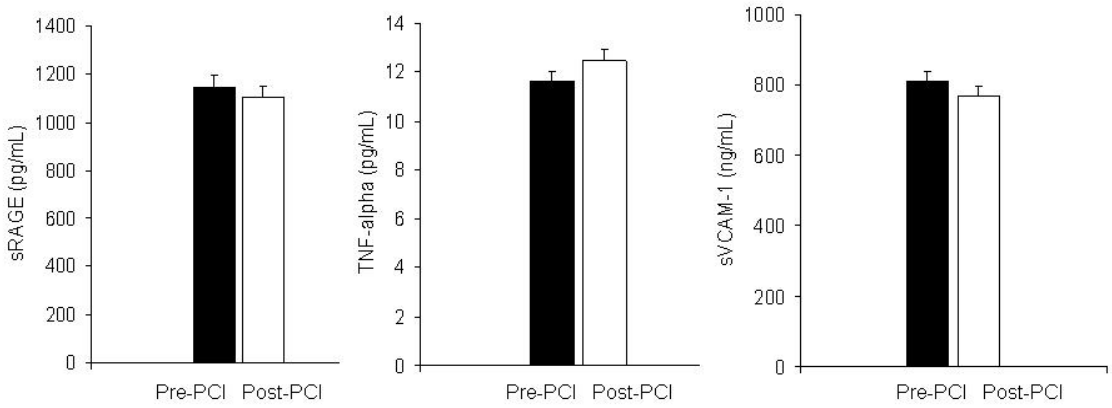


Figure **7.** Pre- and Post-PCI Serum Levels of sRAGE, TNF-α, and sVCAM-1 in NSTEMI Patients without Restenosis. Soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), soluble vascular cell adhesion molecule-1 (sVCAM-1). Pre- and post-procedural percutaneous coronary intervention (Pre-and Post-PCI). Results are expressed as mean ± SE.

4.2.7 Changes in Post-PCI Levels of sRAGE, TNF-α, and sVCAM-1 compared to Pre-PCI Levels Expressed as a Percentage in Patients with and without Restenosis

*Post-PCI Levels of Serum sRAGE as Percentage of Pre-PCI*

Post-PCI levels of serum sRAGE expressed as a percentage of the pre-PCI levels of serum sRAGE for patients with and without restenosis are summarized in Figures 8 and 9. There was a 20% decrease ($p = 0.001$) in post-PCI levels of serum sRAGE as compared to the pre-PCI levels in the restenosis group. However, there was no significant decrease in the post-PCI levels as compared to the pre-PCI levels in the patients without restenosis.

*Post-PCI Levels of Serum TNF-α as Percentage of Pre-PCI*

Post-PCI levels of TNF-α expressed as a percentage of the pre-PCI levels of serum TNF-α for patients with and without restenosis are summarized in Figures 8 and 9. There was an increase of 42% ($p = 0.001$) in the post-PCI levels of TNF-α compared to the pre-PCI levels in patients with restenosis. However, there was no significant increase in the post-PCI levels of TNF-α when compared to the pre-PCI levels in the patients without restenosis.

*Post-PCI Levels of Serum sVCAM-1 as a Percentage of Pre-PCI*

Post-PCI levels of sVCAM-1 expressed as a percentage of the pre-PCI levels of serum sVCAM-1 for patients with or without restenosis are summarized in Figures 8 and 9. There was a no significant increase in the post-PCI levels of sVCAM-1 compared to the pre-PCI levels in the restenosis groups. There was no significant decrease in the post-PCI values compared to the pre-PCI levels in the patients without restenosis.

In summary, the results show that there was a 20% decrease in the levels of serum sRAGE and a 40% increase in serum TNF-α as compared to the pre-PCI levels in the restenosis group. The Pre-PCI levels of serum sRAGE, TNF-α, and sVCAM-1 were similar to post-PCI levels in patients without restenosis.

**Pre-and Post-PCI Levels of sRAGE, TNF-α, and sVCAM-1 Expressed as a Percentage in Patients with Restenosis**

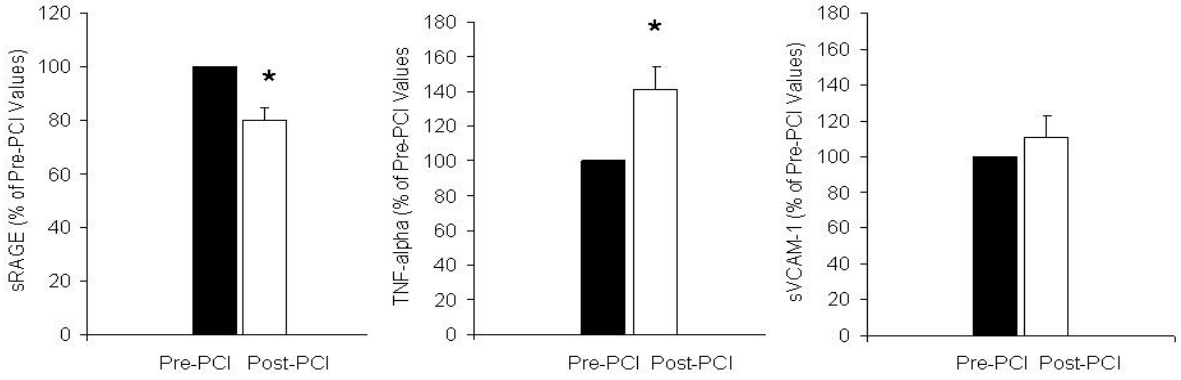


Figure 8. Changes in Post-PCI Levels of Serum sRAGE, TNF-α, and sVCAM-1 compared to Pre-PCI Levels Expressed as a Percentage in Patients with Restenosis. Soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), soluble vascular cell adhesion molecule-1 (sVCAM-1). Pre- and post-procedural percutaneous coronary intervention (Pre-and Post-PCI). Results are expressed as mean ± SE. * $p < 0.05$, Pre-PCI vs. 6 months post-PCI.

**Pre-and Post-PCI Levels of sRAGE, TNF-α, and sVCAM-1 Expressed as a Percentage in Patients without Restenosis**

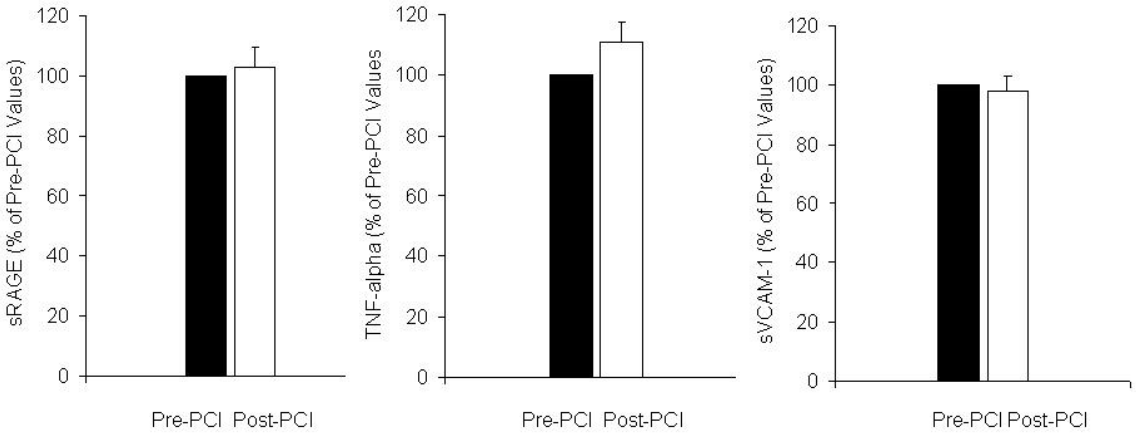


Figure 9. Changes in Post-PCI Levels of Serum sRAGE, TNF-α, and sVCAM-1 compared to Pre-PCI Levels Expressed as a Percentage in Patients without Restenosis. Soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), soluble vascular cell adhesion molecule-1 (sVCAM-1). Pre- and post-procedural percutaneous coronary intervention (Pre-and Post-PCI). Results are expressed as mean ± SE.

4.2.8 Correlation of Pre-PCI and Post-PCI sRAGE to TNF-α and sVCAM-1 in Patients with and without Restenosis

*sRAGE vs. TNF-α in Patients with Restenosis*

The correlation between pre-PCI sRAGE and pre-PCI TNF-α and between post-PCI sRAGE and post-PCI TNF-α in patients with restenosis are depicted in Figure 10. The Spearman correlation coefficients and p values for pre-PCI levels of sRAGE vs. TNF-α were $r = -0.69$ and $p<0.001$, while the correlation coefficients and p values for post-PCI sRAGE vs. TNF-α were $r = -0.55$ and $p<0.001$, respectively. There was an inverse relationship between sRAGE and TNF-α irrespective of pre- and post-PCI status.

*sRAGE vs. sVCAM-1 in Patients with Restenosis*

The correlation between pre-PCI sRAGE and pre-PCI sVCAM-1 and between post-PCI sRAGE and post-PCI sVCAM-1 in patients with restenosis are depicted in Figure 11. The Spearman correlation coefficients and p values for pre-PCI levels of sRAGE vs. sVCAM-1 were $r = -0.60$ and $p<0.05$, while the correlation coefficients and p values for post-PCI sRAGE vs. TNF-α were $r = -0.57$ and $p<0.05$, respectively. There was an inverse relationship between sRAGE and sVCAM-1 irrespective of pre- and post-PCI status.

*sRAGE vs. TNF-α in Patients without Restenosis*

The correlations between pre-PCI sRAGE and pre-PCI TNF-α and between post-PCI sRAGE and post-PCI TNF-α in patients without restenosis are depicted in Figure 12. The Spearman correlation coefficients and p values for pre-PCI levels of sRAGE vs. TNF-α were $r = -0.67$ and $p< 0.001$, while the correlation coefficients and p values for post-PCI sRAGE vs. TNF-α were $r = -0.62$ and $p<0.001$, respectively. There was an inverse relationship between sRAGE and TNF-α irrespective of pre- and post-PCI status.

*sRAGE vs. sVCAM-1 in Patients without Restenosis*

The correlation between pre-PCI sRAGE and pre-PCI sVCAM-1 and between post-PCI sRAGE and post-PCI sVCAM-1 in patients without restenosis are depicted in Figure 13. The Spearman correlation coefficients and p values for pre-PCI levels of sRAGE vs. sVCAM-1 were $r = -0.64$ and $p<0.001$, while the correlation coefficients and p values for post-PCI sRAGE vs. TNF-α were $r = -0.68$ and $p<0.001$, respectively. There was an inverse relationship between sRAGE and sVCAM-1 regardless of pre- and post-PCI status.

In summary, these data demonstrate that the serum levels of sRAGE are negatively correlated with the serum levels of TNF-α and sVCAM-1 irrespective of pre- and post-PCI status.

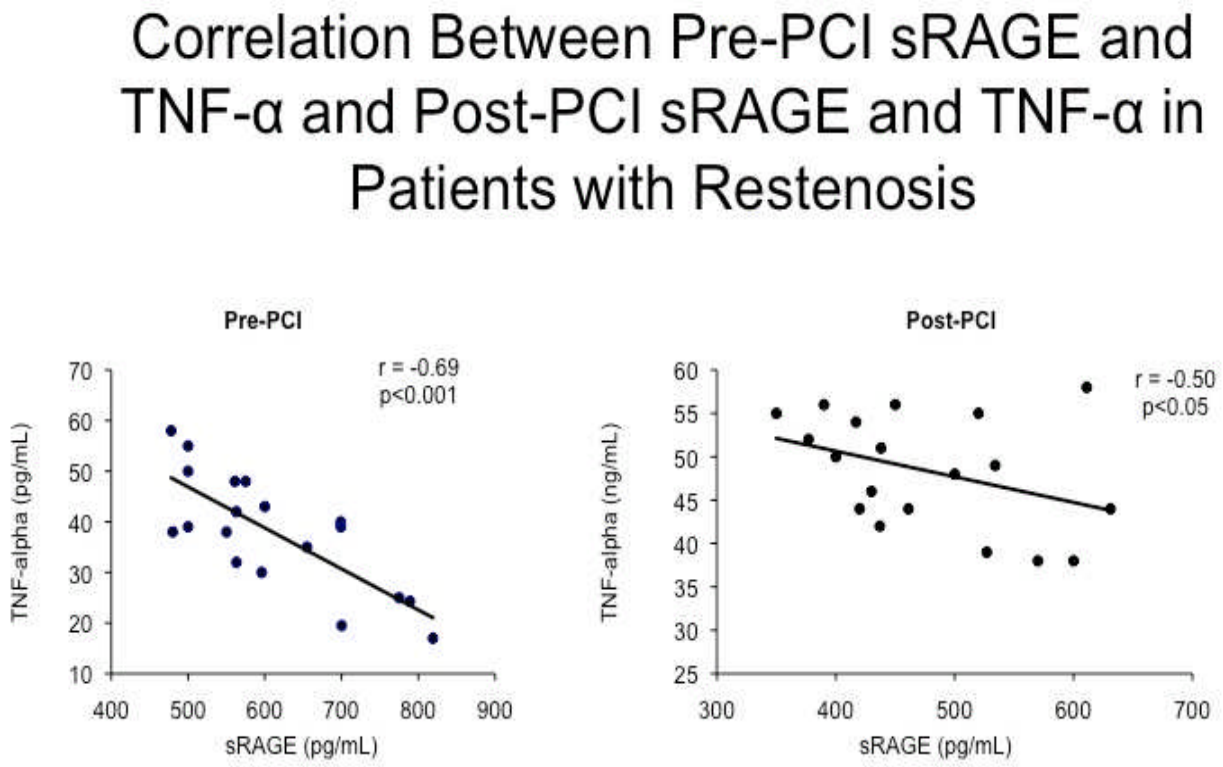


Figure 10. Correlation between sRAGE and TNF-α (pre- and post-PCI) in patients with restenosis. Soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), percutaneous coronary intervention (PCI).

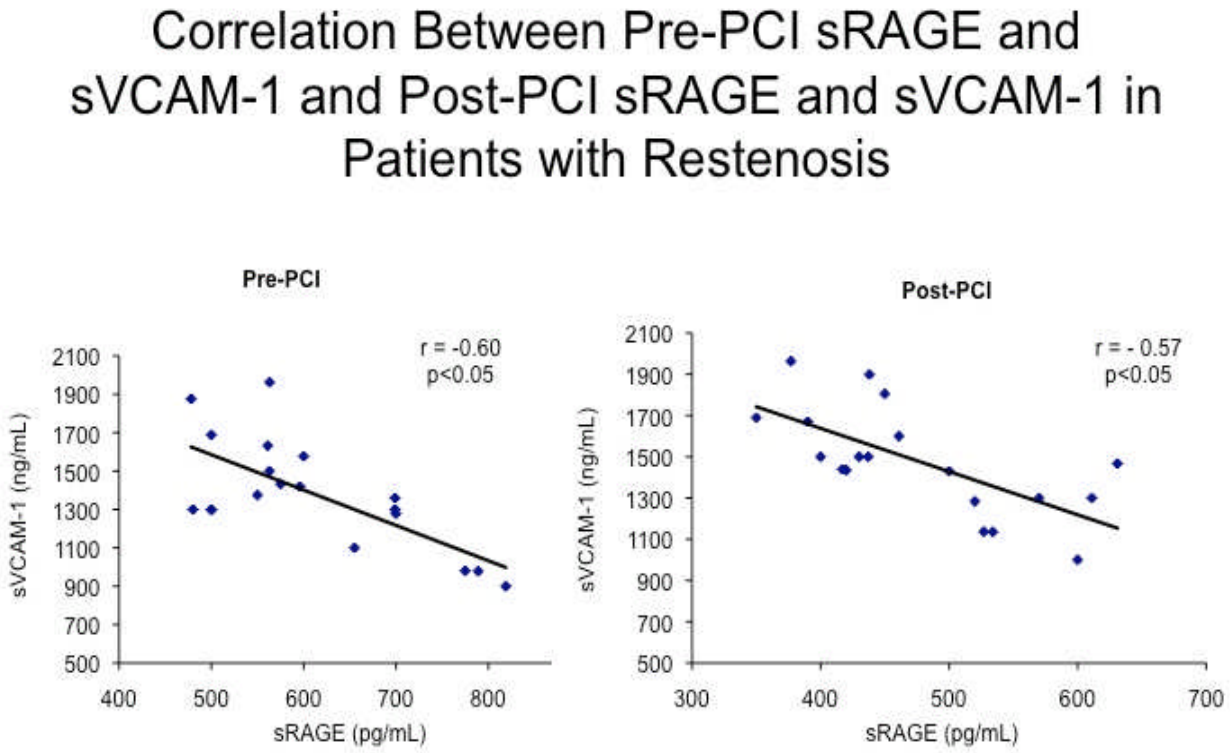


Figure 11. Correlation between sRAGE and sVCAM-1 (pre- and post-PCI) in patients with restenosis. Soluble receptor for advanced glycation end products (sRAGE), soluble vascular cell adhesion molecule-1 (sVCAM-1), percutaneous coronary intervention (PCI).

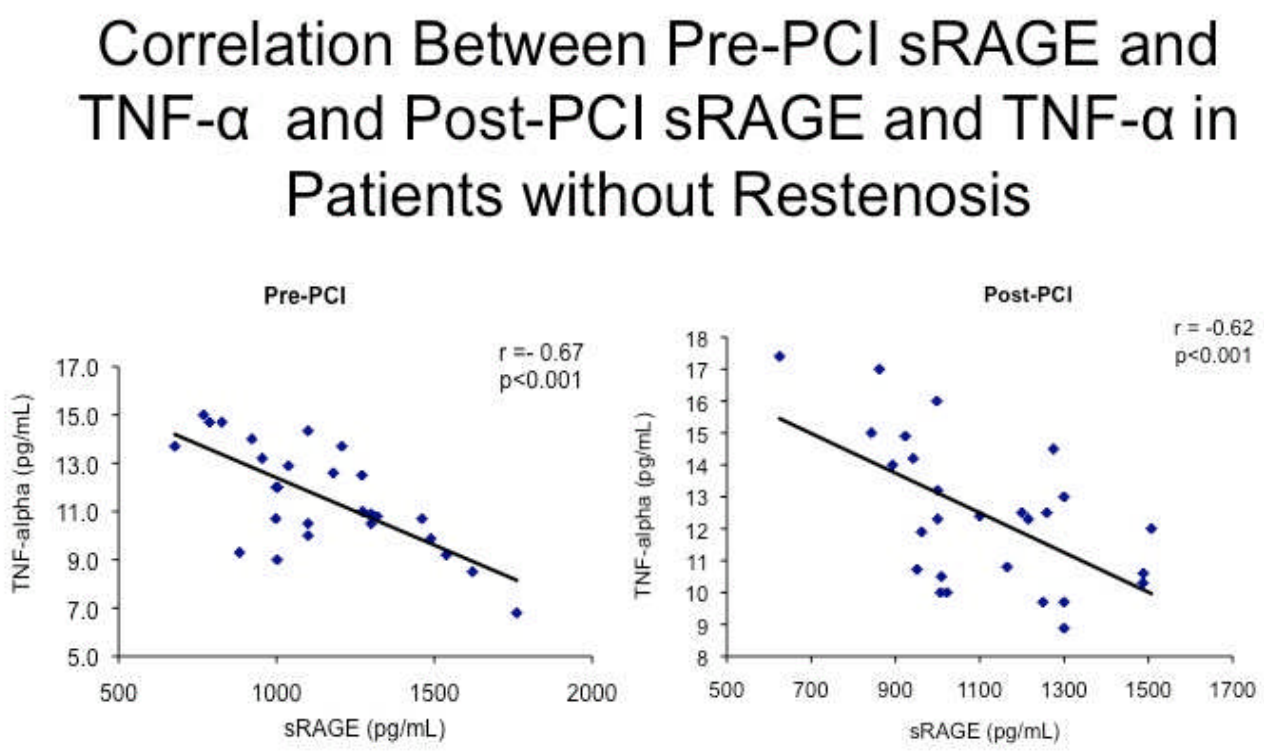


Figure 12. Correlation between sRAGE and TNF-α (pre- and post-PCI) in patients without restenosis. Soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), percutaneous coronary intervention (PCI).

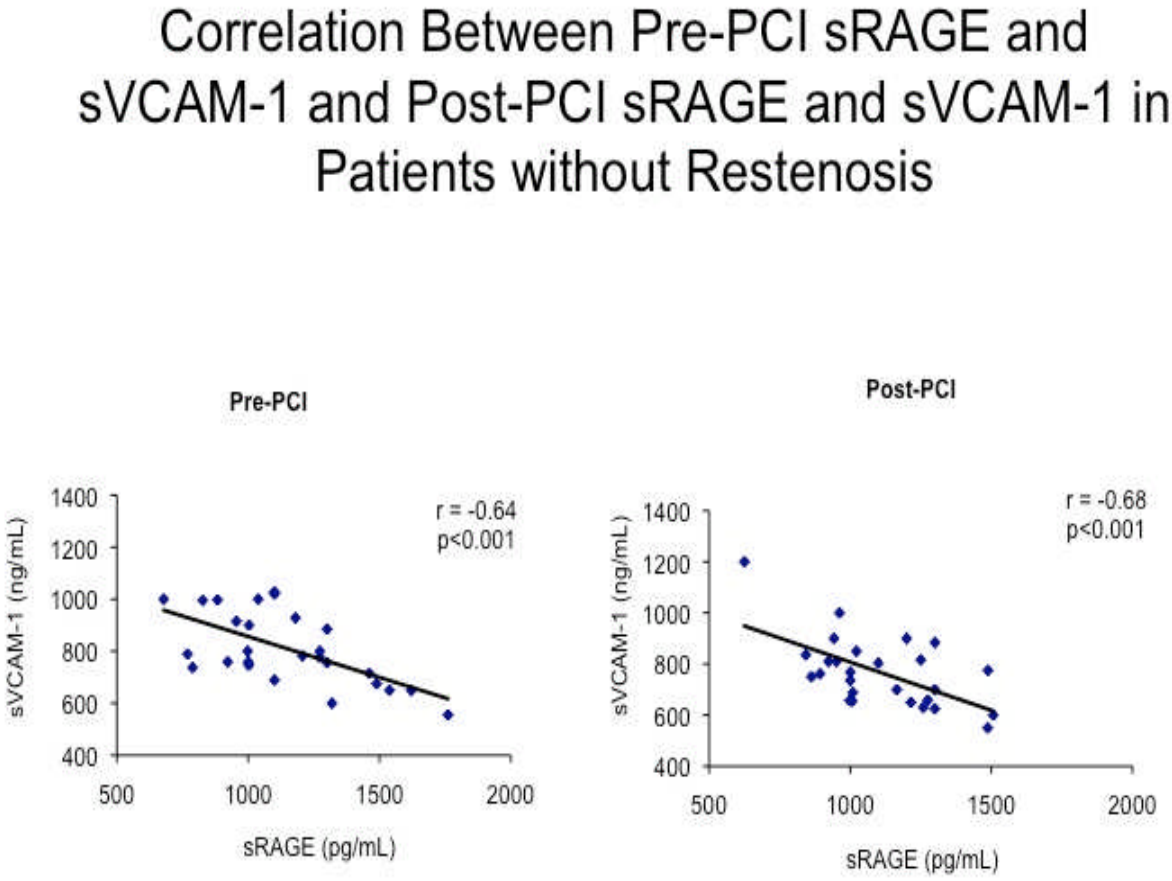


Figure 13. Correlation between sRAGE and sVCAM-1 (pre- and post-PCI) in patients without restenosis. Soluble receptor for advanced glycation end products (sRAGE), tumor necrosis factor-alpha (TNF-α), soluble vascular cell adhesion molecule-1 (sVCAM-1), percutaneous coronary intervention (PCI).

4.2.9 Sensitivity, Specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and Accuracy of sRAGE Tests for Post-PCI Restenosis

The sensitivity, specificity, PPV, NPV, and accuracy of the pre-PCI sRAGE tests were 73%, 100%, 100%, 80%, and 87%, respectively in identifying patients who developed post-PCI restenosis (Table 6). The sensitivity, specificity, PPV, NPV, and accuracy of the AGE/sRAGE tests were 81%, 94%, 93%, 84% and 88% respectively, in identifying patients who developed post-PCI restenosis (Table 6).

In summary, these data show that both sRAGE and AGE/sRAGE tests are valid as a biomarkers/ predictors of patients who may develop post-PCI restenosis. However, the sensitivity of AGE/sRAGE is greater than that of sRAGE as a biomarker/ predictor of post-PCI restenosis.

**Table 6: Sensitivity, Specificity, Predictive Values and Accuracy of sRAGE and AGE/sRAGE Tests for Post-PCI Restenosis in NSTEMI Patients**

| Biomarker | Sensitivity | Specificity | PPV | NPV | Accuracy |
|---|---|---|---|---|---|
| **sRAGE** | **100%** | **83%** | **85%** | **100%** | **91%** |
| **AGE/sRAGE** | **81%** | **94%** | **93%** | **84%** | **88%** |

**S**ensitivity: The probability that a test result will be positive in patients with the disease.

Specificity: The probability that a test result will be negative in individuals without the disease.

Positive Predictive Value (PPV): The probability that a patient will have a disease given a positive test result.

Negative Predictive Value (NPV): The probability that an individual will not have a disease given a negative test result.

Accuracy: The probability of correctly identified subjects.

NSTEMI: Non-ST-elevation myocardial infarction

## 5.0 DISCUSSION

### 5.1 Part I. Serum sRAGE and NSTEMI

**sRAGE**

To date, this is the first study where it has been shown that serum sRAGE levels are lower in NSTEMI patients compared to control subjects, and that the levels are inversely related to the number of diseased vessels. The results also demonstrate a negative correlation between the extent of coronary artery lesions and the levels of sRAGE. Falcone et al (2005) have shown that the concentration of sRAGE in the plasma of patients with CAD was lower {median (inter quartile range): 966 (658-1371) pg/mL] than in control subjects [1335 (936-1954) pg/mL], which was comparable to our values. However, patients with ACS were excluded from this study. Basta et al (2008) have reported that plasma levels of sRAGE were not elevated 1 day post-PCI but significantly elevated at 6 months post-PCI.

***AGE***

The present study has shown that the serum AGE concentrations are higher in NSTEMI patients compared to control subjects, and that the levels are positively correlated with the extent of the coronary artery lesion and negatively correlated with the serum sRAGE levels. Basta et al (2008) have reported that the plasma N-ε-(carboxymethyl) lysine (CML) (AGE adduct) levels were elevated on day 1 and 6 months post-PCI in patients with CAD. Their data suggest that CML is associated with vessel injury post-stenting. However, they did not disclose whether the patients did or did not develop restenosis at six months and whether the levels of CML and sRAGE were associated with restenosis. They also did not compare the pre-stent levels of CML with healthy control subjects. Their study included patients with stable and unstable angina. In another study it was

shown that plasma levels of pentosidine, a marker of AGE, are associated with coronary artery calcification in hemodialysis patients (Taki et al., 2006). These data imply that AGEs are associated with vessel injury which is consistent with our study which shows that AGEs are associated with the extent of vessel injury in NSTEMI patients. Nakamura et al (2007) reported a positive correlation between sRAGE and AGE, which is contrary to the present findings. This difference could be due to the difference in the type of study subjects. The Nakamura subjects were diabetic patients with CAD, while the present study involved male non-diabetic NSTEMI patients. They also reported higher levels of sRAGE in their CAD patients compared to healthy control subjects. Yagamishi et al (2006) have shown that serum sRAGE levels were positively associated with AGEs in the non-diabetic general population. These differences could not be explained at present.

***TNF-α***

In the present study, serum levels of TNF-α and sVCAM-1 were higher in NSTEMI patients compared to control subjects. In addition, there were inverse correlations between sRAGE and TNF-α and sVCAM-1. To date, there have been no similar studies performed investigating NSTEMI patients. However, there are reports of elevated levels of TNF-α in STEMI patients (Gonzalvez et al., 2007; Theroux et al., 2005; Antonicelli et al., 2005). Individuals carrying the TNF-α-308 AG +AA genotypes are significantly more represented among acute myocardial infarction patients affected by STEMI than among NSTEMI and healthy controls (Antonicelli et al., 2005). Correlations of sRAGE with TNF-α in NSTEMI patients have not been reported earlier. However, Nakamura et al (2007) have reported a positive correlation between sRAGE and TNF-α in type II diabetic patients. This discrepancy may be due to the different patient populations.

***sVCAM-1***

In the present investigation, serum levels of sVCAM-1 were elevated in NSTEMI patients compared to health controls subjects, and the levels were inversely related to the serum sRAGE concentrations. This is the first study of this kind in NSTEMI patients. However, levels of serum sVCAM-1 have been reported in STEMI patients. According to Dominguez et al (2008) the serum levels of sVCAM-1 in STEMI patients ranged from 1391 ± 38 and 1200 ± 43 ng/mL. Milosz et al (2007) reported that type II diabetic patients with STEMI had higher levels of serum sVCAM-1 compared to control subjects (1393.4 ± 856.4 *versus* 573.3 ± 226.1 ng/mL). These values are similar to the values reported in the present study with NSTEMI patients and control subjects (1059.6 ± 71 *versus* 651 ± 35.5 ng/mL). However, there is one study in which the levels of serum sVCAM-1 in STEMI patients was 496 ± 34 ng/mL, which is lower than controls in other studies (Stefanidi et al., 2009).

**Sensitivity, Specificity, Positive Predictive Value, Negative Predictive Value and Accuracy of the sRAGE and AGE/sRAGE Test**

The present investigation is the first of its kind to report on the AGE/sRAGE ratio. The AGE/sRAGE ratio in control subjects was 0.52 ± 0.06, while the ratio in NSTEMI patients was 1.75 ± 0.17. The ratio was significantly ($p < 0.001$) higher in NSTEMI patients compared to control subjects. These data reflect the fact that the serum levels of sRAGE are lower and AGE and AGE/sRAGE are higher in NSTEMI patients compared to control subjects.

Since the combination of AGE, RAGE, and sRAGE plays a role in the extent of vascular injury, the measurements of these parameters would be relevant in determining the vascular injury (atherosclerosis). However, in humans serum RAGE is inaccessible to

measurement since it is a cell surface protein on the vascular endothelium. Thus, the levels of serum AGE and sRAGE and the ratio of AGE/sRAGE is important for determining the degree of the vascular lesion and may be used as a biomarker/predictor of vascular complications. If the levels of serum sRAGE are high then there there is less AGE to interact with RAGE and the development of atherosclerosis may be attenuated. However, if the AGE/sRAGE ratio is high more AGE will interact with RAGE to produce adverse effects including vascular lesions. The sensitivity, specificity, PPV, NPV, and accuracy of the sRAGE biomarker test were 59%, 100%, 100%, 100% and 74% respectively, while those of the AGE/sRAGE test were 85%, 91%, 97%, 67% and 86%, respectively, in the diagnosis of patients with NSTEMI acute coronary syndrome. These data suggest that both sRAGE and AGE/sRAGE tests may serve as biomarkers/predictors for identifying patients with NSTEMI. However, the sensitivity of AGE/sRAGE appears to be better than that of sRAGE, while the predictive value of the sRAGE is better than that of the AGE/sRAGE.

## 5.2 Part II. Serum sRAGE and Post-PCI Restenosis

### sRAGE

This is the first study where it has been shown that pre-PCI levels of serum sRAGE are lower in NSTEMI patients who developed post-PCI restenosis compared to those who did not develop restenosis. The data support the findings of Sakaguchi et al. (2003) who reported that administration of sRAGE to experimental animals exposed to arterial injury and developed neointimal expansion had drastically reduced atherosclerosis and restenosis. sRAGE suppresses neointimal growth following arterial denudation by sequestering RAGE ligands and preventing their interaction with cell receptors (Yonekura et al., 2003). In addition, this is the first study to demonstrate that the serum levels of sRAGE are inversely related to the serum levels of TNF-α and sVCAM-1 in NSTEMI patients. Clinical studies in nondiabetic human patients have suggested that increased levels of sRAGE may be correlated with a lower risk of the development of CAD, hypertension and arthritis (Falcone et al., 2005; Pullertis et al., 2005 Geroldi et al., 2005). S100A12, also called ENRAGE (extracellular newly identified receptor for AGE binding protein) is a class of RAGE ligands that can attach to RAGE inducing cellular activation which contributes to vasculature pathology associated with atherosclerosis. ENRAGE has also been identified as a proinflammatory cytokine expressed by granulocytes and is associated with $Ca^{2+}$ dependent signaling (Dell Angelica et al., 1994; Yonekura et al., 2003). Basta et al (2006) have shown that plasma sRAGE concentrations in diabetics are inversely related to the proinflammatory molecules, AGE and S100A12 (ENRAGE). Moreover, Basta et al (2008) have demonstrated low pre-PCI levels of sRAGE in unstable and stable angina patients who underwent PCI [406 (266-757) pg/mL]. These data are consistent with our findings of low levels of serum

sRAGE in NSTEMI patients who develop restenosis. sRAGE may act as a naturally occurring competitive decoy of the signaling pathways induced by the interaction of RAGE ligands with RAGE. Therefore, sRAGE may augment the elimination of circulating RAGE ligands and attenuate restenosis.

***TNF-α***

The current study is the first to demonstrate that serum levels of sRAGE and TNF-α are inversely associated in NSTEMI patients who developed restenosis after stenting compared to those who did not develop restenosis. Currently there are no data available reporting on the association of sRAGE, TNF-α, and restenosis in NSTEMI patients. It is known that elevated serum levels of TNF-α, primarily produced by macrophages, are associated with atherosclerosis and neointimal formation (Blake et al., 2001). In addition to macrophages and monocytes TNF-α is also be produced by endothelial and vascular smooth muscle cells (Blake et al., 2001). The present study demonstrated that levels of serum TNF- α were higher in NSTEMI patients who developed restenosis as compared to those who did not. The present data are in agreement with the results of Caixeta et al. (2007) in which they investigated patients with unstable (U/A) and stable angina (S/A) who underwent PCI. They demonstrated that proinflammatory cytokines (TNF-α, IL-6, IL-8) and inflammatory markers (CRP) are released into the systemic circulation following stenting and that the levels of these markers are positively correlated with the development of post-PCI restenosis. Likewise, Fukuda et al (2004) reported that total blood monocyte count increased after coronary stenting. At 24 hours, monocytes peaked and were correlated with in-stent neointimal volume (plaque burden). Kozinski et al (2005) also found elevated TNF-α levels in U/A and S/A patients at 1-

month post-PCI. Furthermore, they demonstrated that PCI stimulates a systemic inflammatory response with elevated TNF-α levels in patients who developed restenosis.

***sVCAM-1***

The present study demonstrated an inverse relationship between the levels of serum sRAGE and sVCAM-1 in patients with NSTEMI who developed post-PCI restenosis and those results are consistent with the findings of Schmidt et al. (1995 and 1996) in their study of diabetic patients. The AGE-RAGE interaction promotes cellular oxidative stress and activation of the transcription factor NF-κB. Electrophoretic mobility shift assays on nuclear extracts from AGE-treated endothelial cells demonstrated selective binding activity for NF-κB in the VCAM-1 promoter region of DNA. This resulted in increased expression of endothelial VCAM-1 and soluble VCAM-1 to similar degrees. It was further shown that administration of sRAGE blocked the transcription and expression of VCAM-1 and sVCAM-1 (Schmidt et al., 1995; Schmidt et al., 1996). These data suggest that high levels of sRAGE are linked with decreased levels of sVCAM-1. On the contrary, Nakamura et al (2008) reported a positive relationship between sRAGE and AGEs and sVCAM-1 in patients with type II diabetes.

***Post-PCI and Restenosis***

The two major categories of restenosis for PCI (balloon angioplasty and stent implantation) are angiographic restenosis, defined as a less than 50% reduction of lumen diameter at follow-up angiography, which occurs in 25 to 50% of all cases; and clinical restenosis, defined as recurrent angina (chest discomfort) with angiographic restenosis greater than 50% which occurs in 30 to 45% of patients (Hansrani et al., 2002). Clinical in-stent restenosis (ISR) remains a major problem with bare metal stents, occurring in

30-40% of patients following PCI (Erbel et al., 1996; Cutlip et al., 2002). Some investigators report restenosis rates as high as 51% in the bare metal stent group vs. the sirolimus DES (Lemos et al., 2003; Schampaert et al., 2004; Schofer et al., 2003). In the present study the restenosis rate was 41%.

***sRAGE and Atherosclerosis***

The results indicate that low serum levels of sRAGE were associated with high levels of serum TNF-α and sVCAM-1 in NSTEMI patients. Perhaps CAD in these patients may be related to an increase in the levels of serum TNF-α and sVCAM-1. TNF-α stimulates superoxide ($O_2^-$) production in neutrophils and endothelial cells via NADPH oxidase, xanthine oxidase, and nitric oxide synthetase (Sorescu and Griendling, 2002; Downey et al., 1991; Pritchard et al., 1995). TNF-α activates NFκB which controls the expression of genes pertaining to inflammation and oxidative stress (Kumar et al., 2004). TNF-α decreases the bioavailability of nitric oxide by diminishing the production of endothelial constitutive nitric oxide synthetase production (Marczin et al., 1996) and increasing the removal of NO (Gao et al., 2007). Oxidative stress and NO deficiency have been implicated in the pathophysiology of atherosclerotic disease (Blankenberg et al., 2003; Steinberg, 1992; Prasad and Lee, 2007; Cooke et al., 1992).

Adhesion molecules are involved in the genesis and progression of atherosclerosis (Libby et al., 2002; Blankenberg et al., 2003). According to Peter et al. (1997) in patients with known CAD, sVCAM-1 is an acceptable biomarker of the extent and severity of atherosclerosis. The present study also demonstrated a positive correlation between sVCAM-1 and the extent of the plaque burden of the vessel segment in

NSTEMI patients. These results suggest the involvement of sVCAM-1 in coronary artery disease.

Low levels of serum sRAGE in NSTEMI patients may be associated with CAD in these patients. Zhou et al. (2003) reported that the levels of AGEs and RAGE in the carotid arterial endothelium were higher in Zucker diabetic rats compared to euglycemic controls. They induced injury to the carotid arteries in these rats by balloon inflation which further increased the concentrations of AGEs and RAGEs, and produced in neointimal hyperplasia. Treatment with sRAGE before, and up to 21 days post-balloon damage, significantly attenuated the neointimal growth. Sakaguchi et al. (2003) showed that endothelial injury in mice was associated with neointimal expansion and deposition of AGE in the expanding neointimal. Furthermore, sRAGE administration in this study decreased neointimal expansion, and smooth muscle cell proliferation and migration. sRAGE treatment significantly attenuated atherosclerotic lesion and VCAM-1 in their study. Treatment of diabetic apo-E deficient mice with sRAGE completely suppressed atherosclerotic lesion development (Park et al., 1998; Bucciarelli et al., 2002). The present study also demonstrated that sRAGE values are inversely correlated to the lesion volume in NSTEMI patients suggesting that lower sRAGE levels resulted in increased plaque burden of NSTEMI patients. The present study results showed a positive correlation between AGE and the extent of vascular injury. These results are consistent with those of Basta et al. (2008) who reported a positive correlation between plasma N-ε-(carboxymethyl) lysine adducts and the degree of lesion development in patients' post-PCI. The results of this study demonstrate that patients with NSTEMI with restenosis had lower levels of serum sRAGE and higher levels of serum TNF-α and sVCAM-1

compared to those without restenosis. Furthermore, the results demonstrate that there is a negative correlation between levels of sRAGE and TNF-α and levels of sRAGE and sVCAM-1. Consequently, low levels of serum sRAGE may promote the development of restenosis through an increase in the levels of serum TNF-α and sVCAM-1. Low levels of serum sRAGE would favor increased AGE to interact with RAGE. The RAGE-AGE interaction in SMC may lead to the release of growth factors, resulting in SMC migration, proliferation and extracellular matrix secretion and thus restenosis.

Based on the work achieved and background information a possible mechanism of atherosclerosis/ restenosis is proposed (Figure 14).

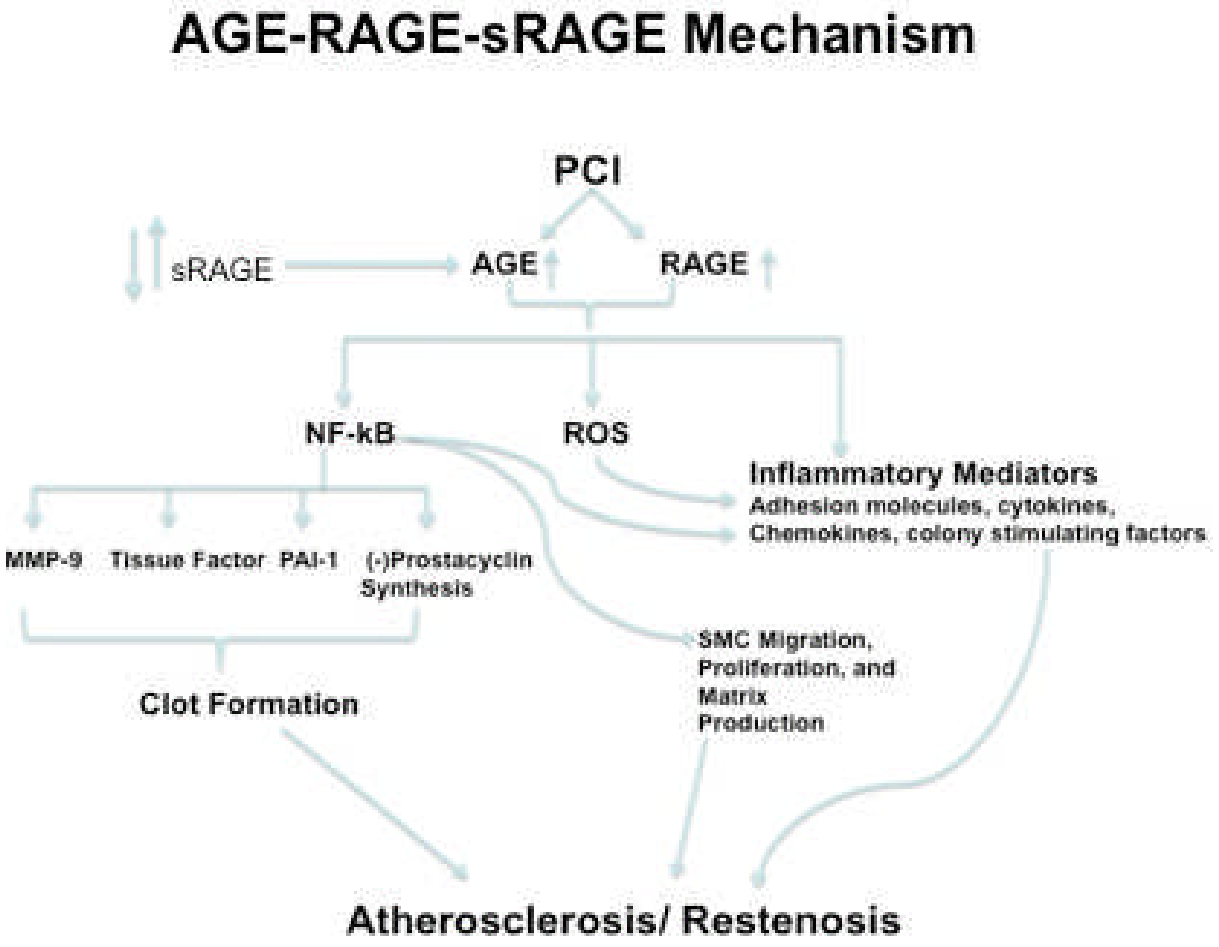


Figure 14. The mechanism by which the AGE-RAGE interaction induces atherosclerosis/ restenosis following injury from percutaneous coronary intervention. PCI, percutaneous coronary intervention, AGE, advanced glycation end products, RAGE, receptor for advanced glycation end products, ROS, reactive oxygen species, MMP-9, metalloproteinase-9, SMC, smooth muscle cell, PAI-1, plasminogen activator inhibitor-1.

### 5.3 Limitations of the Study

There are some limitations in this study. (1) The sample size is small however; the results were clear-cut. Although sRAGE and AGE/sRAGE were strong and independent discriminators between NSTEMI and control groups, there was an overlap in the levels of the two groups. A larger sample size may have corrected this problem. (2) The presence of underlying coronary artery disease cannot be ruled out because coronary angiograms were not performed on the control subjects. (3) A study with a larger sample size of patients and a longer duration of follow up would be desirable. A longer follow up might have detected restenosis in patients who showed no restenosis at 6 months. (4) When the study was initiated the only method available for the measurement of sRAGE was the measurement total sRAGE. In this study the method used could not discriminate between sRAGE that was enzymatically cleaved from cell surface RAGE and secretory splice variants of RAGE. (5) The correlation study of sRAGE with lesion volume is controversial. For the measurement of lesion volume we assumed that the lesion was uniform and cylindrical. Under real life circumstances this is not always true. (6) Measurement of sRAGE, AGE, sVCAM-1 and TNF-$\alpha$ at frequent intervals up to one-year post-PCI would have provided more information. This was not possible because of inconvenience to patients as well as the cost. (7) The present data cannot be generalized to females, other ethnic groups and age groups.

Although there are several limitations to the study, to date, the findings are unique and original. The data stress the importance of sRAGE and AGE/sRAGE to be a biomarker/ predictor for NSTEMI, ACS and for post-PCI restenosis.

## 5.4 Significance of the Study

The data from this study suggests that serum levels of sRAGE and AGE/sRAGE ratio are predictors/ biomarkes of NSTEMI. If the data are true clinicians could test patients for AGE and sRAGE during their annual physicals and if the serum sRAGE levels are low or the AGE/sRAGE ratio is high then further investigations for CAD would be needed (exercise tolerance test).

If the data of this study are correct (sRAGE and AGE/sRAGE are predictor/ biomarkers for restenosis) patients that fit the criteria for a PCI could be pre-tested for AGE and sRAGE. This will also open avenues for therapeutic (administration of RAGE blocking agents aminoguanidine or aldose reductase or sRAGE) targeting in the prevention of CAD.

## 6.0 Conclusion

### 6.1 Part I. Serum sRAGE and NSTEMI

The data suggest the following: (1) the levels of serum sRAGE are lower while the serum levels of AGE, sVCAM-1 and TNF-α are higher in subjects with NSTEMI as compared to healthy controls; (2) levels of serum sRAGE are inversely related to the number of diseased vessels and lesion volume while that of AGE, sVCAM-1, and TNF-α are positively correlated with the number of diseased vessels and lesion volume; (3) levels of serum sRAGE are inversely related to AGE, AGE/sRAGE, TNF-α and sVCAM-1 both in healthy and NSTEMI subjects; (4) the sensitivity, specificity, positive predictive value, negative predictive value and accuracy of the sRAGE biomarker test were 59%, 100%, 100%, 100% and 74% respectively; (6) the sensitivity, specificity, positive and negative predictive values and accuracy of the AGE/sRAGE test were 85%, 91%, 97%, 67% and 86%, respectively. (7) the sensitivity and accuracy of AGE/sRAGE tests appears to be greater than sRAGE while the predictive value of sRAGE is greater than AGE/sRAGE; and (8) sRAGE levels may serve as a new biomarker/predictor for the diagnosis of patients with NSTEMI acute coronary syndrome.

### 6.2 Part II. Serum sRAGE and Post-PCI Restenosis

The data suggest the following: (1) the pre-PCI levels of serum sRAGE are lower while that of AGE, AGE/sRAGE, sVCAM-1 and TNF-α are higher in NSTEMI patients with post-PCI restenosis as compared to those without post-PCI restenosis; (2) the post-PCI levels of serum sRAGE are lower and TNF-α are higher than the pre-PCI levels in patients with restenosis; (3) pre- and post-PCI levels of serum sRAGE, TNF-α and sVCAM-1 were similar in patients without restenosis; (4) the sensitivity, specificity,

positive predictive value, negative predictive value and accuracy of the sRAGE test in determining patients with post-PCI restenosis were 100%, 83%, 85%, 100%, and 91% respectively while the sensitivity, specificity, positive predictive value, negative predictive value and accuracy of the AGE/sRAGE test in identifying patients with post-PCI restenosis were 81%, 94%, 93%, 84% and 88% respectively and; (5) low pre-PCI levels of sRAGE and a high ratio of AGE/sRAGE may serve as a predictor/ biomarker of post-PCI restenosis.

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